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MSS. for the successive numbers of Vol. 27 should be in the hands of the Editors before the 1st of January, March, May, July, September and November respectively. Contributors are specially requested to arrange references in the manner uniformly adopted in this Journal.

OBITUARY NOTICE.

JOHN DEMPSTER STIRLING.

(1907–1933.)

John Dempster Stirling died on February 12th, 1933, at the early age of twenty-six. Stirling was educated at Kilmarnock Academy, and subsequently proceeded to Glasgow University, where he graduated in 1928 with First Class Honours in Biochemistry. He was appointed by the Senate of the University to the Barbour Research Scholarship, which he held for three years. During the tenure of this Scholarship he studied at the Institute of Physiology, Glasgow University, at the Institute of Physiological Chemistry of the University of Tübingen, and at the Institute of Medical Chemistry of the University of Graz. As a result of his work he was awarded the degree of Ph.D. by the University of Glasgow.

In 1931, Stirling was appointed Biochemist to the Hannah Dairy Research Institute, an appointment which he held until his death. He published, with J. H. Blackwood, a valuable series of papers on the absorption of milk precursors by the mammary gland and on the energy relationships accompanying milk secretion. With G. M. Wishart he carried out a study of the hydrolysis of caseinogen by pepsin and trypsin, the results of which have shed new light on the enzymic cleavage of this protein. Just before his last illness he undertook, with J. H. Blackwood, a comprehensive survey of published literature relating to the effect of pasteurisation on the nutritive value of milk. In addition, Stirling had carried out research on oxidative processes with special reference to those taking place in the living organism. It is hoped that it will shortly be possible to publish the results of this work.

Stirling possessed to a remarkable degree the curiosity, initiative and enthusiasm for investigation which marked him out as a research worker of great promise. He was a delightful companion and a loyal colleague, and his loss will be deplored by a wide circle of friends and co-workers.

N. C. W.

LXXXI. URICASE AND ITS ACTION.

VI. DISTRIBUTION IN VARIOUS ANIMALS.

By RICHARD TRUSZKOWSKI and CELINA GOLDMANÓWNA.

From the Biochemical Laboratory, Faculty of Medicine,
The University, Warsaw.

(*Received March 10th, 1933.*)

Little systematic work on the distribution of uricase in the individual organs and tissues of various animals has been done. Batelli and Stern [1909] examined certain organs of a number of domestic animals; in all cases the liver was found to contain uricase, and in certain cases the kidneys; uricase was not found in the lung, spleen, pancreas, brain or muscle of certain of these animals (horse, dog, cat, rabbit, ox, sheep). Galeotti [1911] reported that the liver of selachians contained uricase, and Przyłęcki [1926; 1927] studied its distribution amongst the invertebrates, without, however, determining in which organs it was present.

Experimental.

The animals examined were dissected immediately after death, the organs taken being freed as far as possible from non-specific tissues (fat, connective tissue, blood, *etc.*). In most cases 2 g. of fresh organ were weighed out and shaken for 60 min. with 20 cc. of 0·025–0·05 % uric acid (as lithium salt, at p_H 8) in the presence of 1 cc. of toluene; in the case of small animals, the smaller organs of several individuals were pooled to make up the required weight. The sample of tissue was ground in a mortar with a few cc. of the uric acid solution, the remainder of which was used to wash the contents of the mortar into the reaction flask. Uric acid was determined colorimetrically by the method of Folin and Denis in the above systems, and in systems containing 20 cc. of uric acid solution and 1·5 cc. of water (this being roughly equivalent to the water content of the added tissues).

The results (Table I), calculated as mg. of uric acid oxidised per g. of fresh tissue, indicate that the sole organs possessed of uricolytic activity in widely distributed species of animals are the liver and/or kidneys. In most cases only the liver contains uricase (horse, sheep, cat, rabbit, guinea-pig, mouse, hedgehog, bat, carp, crayfish, fresh-water mussel); in the ox uricolysis is performed chiefly by the kidney; in the frog the activity of the kidney is approximately equal to that of the liver; whilst in dogs, swine and rats kidney tissue has comparatively little activity. In the case of dogs, the activity found for liver and kidney tissues varies considerably with the breed and, possibly, with the individual, as in one case 10·8 mg. were oxidised per g. of kidney and in another 2·3 mg., whilst, as was stated in Part v, dog-kidneys may be quite free of uricolytic action. It is, further, well-known that certain breeds (Dalmatians) excrete both allantoin and uric acid [Benedict, 1916], and in these dogs it may be assumed that uricase is deficient in either the liver or kidneys, or both of these organs.

Table I.

		Mg. of uric acid decomposed by shaking 20 cc. of 0·025–0·05 % uric acid for 60 min. with 1 g. of tissue												
Animal	Temp.	Liver	Kidney	Skeletal muscle	Heart	Lungs	Brain	Intestine	Stomach	Spleen	Ovary	Testicle	Uricolytic index	Remarks
Ungulata:														
Horse	18°	2·0	0	0	0	0	0	—	—	0	—	0	88	Pancreas inactive
Ox	15°	2·6	8·8	0	—	0	0	—	—	0	—	—	93	
Pig	18°	3·5	1·3	0	—	0	0	—	—	0	—	—	98	
Sheep	18°	2·8	0*	0*	—	0*	—	—	—	0*	—	—	80	
Carnivora:														
Dog (Alsatian)	16°	12·2	10·8	0	0	0·1	0	0	0	0·1	—	0	98	Pancreas inactive
Dog (Fox-terrier)	16°	—	2·3											
Cat	18°	4·5	0	0	—	—	0	0	0	0·1	—	0	97	
Rodentia:														
Rabbit	16°	3·9	0	0		0	0	—	0	—	0	—	95	
Rabbit (foetus, 7 mm.)	16°	3·9												Foetuses, without liver, and placenta inactive
Guinea-pig	16°	2·4	0	0	0	0	0	0	0	0	—	0	94	Suprarenal glands and pancreas inactive
Rat (albino)	16°	3·4	0·6	0	0	0	0	0	0	0	—	0	96	
Mouse (albino)	18°	2·3	0	0	0	0	0	0	—	0	—	0	—	
Insectivora:														
Hedgehog	16°	3·8	0	0	0	0	0	0	0	0	—	0	—	Thyroid and suprarenal glands inactive
Bat (*Eptesicus serotinus* Schreib.)	20°	0·8	0	0	0	0	0	0	—	0	—	0	—	
Various:														
Frog (*R. escul.*)	18°	2·7	2·1	0	0	0	0	0	—	0	0	0	—	Skin and bones inactive
Carp	18°	1·1	0	0	—	—	—	0	—	—	0	—	—	Skin, bones and gills inactive
Crayfish	18°	2·2	0	0	—	—	—	0	—	—	0	—	—	Gills inactive
Fresh-water mussel (*Anodonta mutabilis*)	18°	0·4	—	—	—	—	—	—	—	—	—	—	—	Remainder after removal of liver inactive

* According to Batelli and Stern.

The rabbit examined was a pregnant doe, containing a number of foetuses, 7 mm. in length. It is of interest that the foetal livers had the same uricolytic activity as the maternal liver, and that the remaining tissues were, as in the adult animal, inactive.

Certain of the results found for poikilotherms are in disagreement with those obtained by Przyłęcki [1926; 1927], who was unable to demonstrate uricase in carp-liver and in crayfish, whilst *Anodonta* apparently contained considerable quantities of uricase. The uricolytic action of carp-liver is extremely feeble, and in systems which, as in Przyłęcki's experiments, were not shaken, it would probably not be possible to demonstrate uricase; the same applies to crayfish, in which only the liver is active, whilst Przyłęcki examined the action of a mixture of the tissues of the whole animal. As to fresh-water mussels, Przyłęcki based his statement chiefly on the results of metabolism experiments on the living animals—under these conditions accompanying bacteria were in all probability the cause of the disappearance of injected uric acid.

If the mammalian tissues examined be placed in order of decreasing uricolytic activity, the following series is obtained: ox-kidney > dog-liver > dog-kidney > cat-liver > rabbit-liver > hedgehog-liver > pig-liver > rat-liver > sheep-liver > ox-liver > guinea-pig-liver > mouse-liver > horse-liver > bat-liver. This series differs in several respects from that given by Batelli and Stern, amongst others, in that they placed horse-liver above all other organs except ox-kidney, and found horse-kidney also to possess considerable activity. It is our opinion that these discrepancies should be ascribed not necessarily to faulty experimental technique, but rather to the same causes as those suggested previously for dog-kidneys, namely, to differences in the various breeds of the same species, which are exceptionally prominent in the case of domestic animals. Further, individual differences may exist even amongst different individuals of the same breed, due to pathological conditions in the organs in question, which would, in the case of horses, be the more probable in that normal, healthy animals are not as a rule available in slaughter-house material.

No connection appears to exist between the distribution of uricase and the phylogenetic relationships of the animals examined.

SUMMARY.

1. Uricase is present in the liver of every animal examined.
2. It is also present in the kidneys of oxen, pigs, dogs, rats and frogs.
3. It is absent from the kidneys of the horse, sheep, cat, rabbit, guinea-pig, mouse, hedgehog, bat, carp and crayfish, and from all organs other than the liver and kidneys of all animals examined.

The authors have pleasure in acknowledging their indebtedness to Prof. St J. Przyłęcki, for his help and advice in the prosecution of this research.

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LXXXII. THE ENDOCRINE FACTORS CONCERNED IN THE CONTROL OF THE OVARIAN CYCLE.

I. *XENOPUS LAEVIS* AS A TEST ANIMAL.

By CHARLES WILLIAM BELLERBY.

Department of Social Biology in the University of London.

(*Received March 10th, 1933.*)

Experimental studies upon the relation of the pituitary gland to the ovarian cycle in mammals involve three principal difficulties:

(i) that of removing the pituitary itself without injury to the animal;

(ii) the practical impossibility of removing the anterior lobe without removing or damaging the posterior lobe;

(iii) the necessity of killing the test animal, in most cases, to confirm whether injected extracts or transplants have produced ovulation or other changes in the ovaries.

None of these objections arises, however, in the case of certain amphibia. In a recent series of papers by Hogben [1930], Hogben, Charles and Slome [1931] and Zwarenstein and Shapiro [1933] reporting work carried out on the African Clawed Toad (*Xenopus laevis*) it has been shown:

(i) that ovarian atrophy follows removal of the anterior lobe alone;

(ii) that injection of acid extracts prepared by the method elsewhere described by the writer [Bellerby, 1929] will induce the extrusion of eggs during the period of the year when the animal does not breed either in its natural surroundings or in the laboratory;

(iii) that the characteristic colour response of the animal serves as an infallible indication as to the completeness of removal of either one or both lobes of the pituitary.

The facts that hypophysectomy involving one or both lobes can be carried out in a few minutes with a negligible mortality and a high degree of accuracy, and that the extrusion of the amphibian egg is a sufficiently obvious and unequivocal phenomenon justify the attempt to explore the use of *Xenopus laevis* as a test animal for assay purposes in investigations directed to extend knowledge concerning the chemical properties of anterior lobe hormones and related substances and improve existing methods of extraction, *etc.*

Before ovulation could be regarded as adequate for practical purposes several points which had not been dealt with by the workers already quoted required thorough investigation. These were in particular the reliability of the test, its time relations, and its sensitivity.

Reliability of Xenopus laevis.

The question of reliability demands special attention, because it is well known that a considerable percentage of failures is usually recorded in mammalian experiments. Firstly, there is no danger of error arising in the case of

Xenopus through spontaneous ovulation. *Xenopus* does not ovulate even during the breeding season, when kept under laboratory conditions (Hogben, Zwarenstein). In any case ova extruded after pituitary injection possess no mucilaginous envelope, so characteristic of the amphibian egg when ovulation occurs in the normal way. If the animals are well fed and kept in a warm, well-lighted laboratory the ovaries remain in a healthy condition with black pigmented ova (Hogben) indefinitely. On the other hand unfavourable conditions of captivity (darkness in particular and apparently a temperature lower than that of the animal in its natural surroundings) lead to a retrogression of the ovaries after 4 or 5 months (Zwarenstein and Shapiro). In these circumstances no ripe ova are present in the ovaries, and the animals are, in consequence, unsuitable for experimental work, when results are demanded from a single injection of extract.

The first experiments undertaken by the writer were carried out on animals which had been kept for some months in the laboratory, in a cold underground room, and many failures to produce ovulation with single injections were recorded. Table I shows such an experiment. The acid extracts used in this and all subsequent experiments were prepared in the following way. The glands were collected at the abattoir and dissected out within 4 hours of death. The anterior lobes were weighed and ground with sand in a mortar. The paste was extracted with 1·5 times its weight (*i.e.* original tissue) of 1 % acetic acid for 24 hours at room temperature. The mixture was then centrifuged and the clear extract decanted from the residue and carefully neutralised with 50 % NaOH, using phenol red as indicator, so that the p_H of the fluid was eventually about 7·4. On removing the precipitate by centrifuging a second time, the extract was ready for immediate use. Injection was made into the dorsal lymph sac to leave no doubt concerning the transport of the active substance in the extract to the ovaries *via* the circulation. The final number of cc. of extract obtained was usually greater than g. of tissue taken. In all cases therefore sufficient volume of extract was injected to ensure that the equivalent of a definite amount of tissue was administered, *i.e.* 0·25 g., 0·5 g. or some simple multiple of these amounts.

Table I.

Dosage*	No. of toads used	No. ovulating	Mean no. of eggs shed
0·5	8	0	0
1·0	8	4	17
2·0	8	2	19

* Equivalent g. of original tissue.

In the experiments recorded in Table I, it will be seen that a large number of animals failed to give the expected response of ovulation. Furthermore, a better response was given by the batch which received the intermediate dose and not the maximum dose. These results suggested that some factor or factors, in addition to the potency of the extract was involved. This was probably the condition of the ovaries.

Two series of experiments were carried out to ascertain the cause of failure. In the first 24 toads were selected and divided into 2 batches of 12 each. One batch (A) had all ovulated previously after injection. The other batch (B) consisted of animals which had not done so. All toads received a dose of extract equivalent to 2 g. of fresh gland. In series (B) 2 out of 12 gave a positive response. In series (A) 8 out of 12 gave a positive response. Complete results are given in Table II.

Table II.

No. of eggs shed	
Batch A	Batch B
263	0
8	11
0	0
303	0
10	0
479	12
0	0
540	0
0	0
38	0
0	0
91	0
Total no. of toads ovulating = 8	Total no. of toads ovulating = 2

In the second experiment, recorded in Table III, the non-ovulating animals were killed to ascertain the reason for failure to give the response. It will be seen that 5 animals out of 11 failed to respond to an injection of 2 cc. of extract equivalent to 2 g. of tissue. When killed and examined these animals alone were found to have atrophic ovaries.

Table III.

No. of toad	No. of eggs shed	Condition of ovary when killed
A	0	Ovary atrophic
B	0	,, ,,
C	294	,, normal
D	34	,, ,,
E	0	,, atrophic
F	392	,, normal
G	466	,, ,,
H	64	,, ,,
I	236	,, ,,
J	0	,, atrophic
K	0	,, ,,

From these experiments it became evident that the condition of the ovaries was a deciding factor if perfect reliability was to be guaranteed, and that it was necessary to pay special attention to this point. Subsequently to these experiments all toads to be used were removed to a warm, well-lighted room, and, by only using animals that had been tested for a positive response, it was found possible to approach 100 % reliability in subsequent experiments.

A further issue of considerable importance in safeguarding a high standard of reliability for experiments involving the injection of extracts concerns the relation of degree of absorption of the injected extract to its volume and salt concentration. In Table IV an experiment which only yielded a positive response in 5 out of 12 animals is recorded. The dosage was, in this case, equivalent to 1·0 g. of fresh gland substance in 4·4 cc. of fluid. It was evident from the greatly distended lymph sac that the fluid was not absorbed in the majority of the animals. This was tested by an incision of the skin from which the fluid squirted out under pressure. Even after 3 days the 7 animals which failed to ovulate showed that the extract was still largely unabsorbed (Table IV). That failure to ovulate was not due to atrophic ovaries was ascertained by autopsy.

These experiments show that it is very important to use as small a volume of fluid as is practicable and to avoid methods of preparing extracts which will

Table IV.

No. of toad	No. of eggs shed	Whether extract absorbed	Condition of ovaries of toads killed
P	17	+	—
Q	0	0	Normal
R	82	+	—
S	87	+	—
T	0	0	Normal
U	0	0	,,
V	0	0	,,
W	264	+	—
X	0	0	Normal
Y	0	0	,,
Z	0	0	,,
A 1	37	+	—

ultimately yield a fluid containing a high salt concentration, which will, in consequence, differ largely in osmotic concentration from the lymph and body fluids.

Time relations of the response to single injections.

The chief advantage of *Xenopus* as test animal for work involving assay of pituitary extracts is that extrusion of eggs follows rapidly after introduction of active preparations into the lymph sac. It generally begins about 12 hours and lasts till about 24 hours after injection. The results of a number of experiments, in which the time relations were noted carefully, are given as a frequency distribution in Table V. In these experiments, as in others, toads were kept in separate containers and were examined for the presence of eggs at 12 hourly periods after injection. The eggs found at the end of each period were removed and counted. This was a necessary precaution in determining number of eggs shed, since it was observed that the toads frequently ate them, if they were left in containers for more than 24 hours.

Table V. *Time relation of ovulation following injection.*

Hours	0–12	12–24	24–36	36–48	48–60	60–72	Total no. of cases
Eggs first extruded	1	49	14	4	3	0	71
Completion of ovulation	0	18	17	9	3	0	47

Sensitivity of the response.

Either of two criteria might be selected as a basis for comparing the amounts of gonad-stimulating substance in extracts. One is the number of eggs laid. The other is whether any eggs are laid or not. The first, however, is not a very reliable index of the dosage, for a great variation exists in the number of eggs shed per toad, even when receiving the same amount of extract. For instance, in one experiment embracing 10 toads the number of eggs shed varied per individual from 3 to 1539. In Table VI will be seen results obtained on 76 animals. The figures form a frequency distribution with unequal class intervals.

Table VI. *Frequency distribution of eggs laid.*

0–25	25–75	75–175	175–375	375–725	725–1575
20	15	16	11	10	4

The second criterion gives results, however, which are consistent with the view that response varies with the potency of the extract injected. In the series of experiments recorded in Table VII 3 different series of toads received the same volume of extract but corresponding to different amounts of fresh tissue. The 3 series are combined and the mean number of eggs and range for each batch are given together with the percentage positive response.

Table VII.

Dosage	No. of toads used	No. of toads ovulating	% of toads ovulating	Extremes of eggs laid	Mean no. of eggs shed
0·25	8	0	0	0	0
0·50	21	3	14	53–85	63
1·00	22	5	22	33–285	228
1·50	20	11	55	7–494	112
2·00	16	11	68	15–1062	206
2·50	10	10	100	60–423	247

It will be seen that there is no relation between number of eggs shed and dosage, but that there is a distinct relationship between percentage response and the latter. The data contained in Table VII enable a provisional definition of a unit for use in comparative work to be made. For instance, if the minimum amount of extract is chosen which will produce a response in 50 % of 10 toads, of an average weight of 35 g., this amount of extract would contain 1 toad unit. That is to say 1 kg. of fresh tissue would contain 750 units.

The chief point of practical interest which emerges from these experiments is that there is a much more constant relation between dosage and response in *Xenopus* than the writer has been able to establish using mammals as test animals. Further, the same set of animals may be used repeatedly for assay work. This is advantageous, not merely because it diminishes expense, but also, because it ensures that the ovaries are in good condition. In Table VIII is recorded an experiment in which four successive injections were made on the same batch of animals, at fortnightly intervals.

Table VIII.

No. of toad	No. of eggs shed after successive injections			
	First	Second	Third	Fourth
36 H	423	403	156	294
36 S	169	87	15	392
36 W	110	264	0	236
36 A	131	17	1062	765

CONCLUSIONS.

It would be premature to give full details of the chemical work which has been carried out using *Xenopus laevis* as a test animal. Three facts which have emerged from the present enquiry, at this stage, throw light on the property of anterior lobe extracts. The first two are merely practical. If glands are dissected immediately after killing, the anterior lobes can be kept at a temperature about freezing point for several weeks without affecting greatly the potency of extracts subsequently made from them. A second fact of some interest, in view of the very large quantity of glandular material which is necessary to evoke ovarian changes both in *Xenopus* and in the mammal, is that a subsequent extraction of the solid matter which is separated after centrifuging, for the first time, yields an extract which may have a potency equal to a third of that of the original extract. A third point is of more general interest. In the case of the rabbit, acid extracts prepared by the writer's method and alkaline extracts prepared in the way prescribed by Evans and Long [1921; 1922] both induce ovulation and both induce luteinisation on injection. Experiments on *Xenopus*, made with alkaline extracts, show that these are almost as effective as acid extracts in producing ovulation.

SUMMARY.

1. Acid extracts of ox anterior pituitary, when injected in sufficient quantity into females of *Xenopus laevis* with healthy ovaries, uniformly produce extrusion of eggs.

2. Ovulation usually occurs within 18 hours and is rarely delayed beyond 48 hours.

3. The same animals may be used successively for the assay of extracts.

4. There is a more constant relation between dosage and response than is obtained when the mammal is used as test material.

The cost of animals and glands in this research was borne from a grant made to the Department of Social Biology by the Sex Hormones Committee of the Medical Research Council. Acknowledgment is made to Prof. Lancelot Hogben, who suggested this enquiry.

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LXXXIII. THE GONADOTROPIC HORMONES (ρ-FACTORS).

IV. THE PREPARATION OF EXTRACTS FOR CLINICAL USE, TOGETHER WITH OBSERVATIONS ON THE STABILITY IN SOLUTION AND STANDARDISATION PROCESSES.

By PHILIP GUY MARSHALL.

From the Macaulay Laboratory, Institute of Animal Genetics, University of Edinburgh.

(*Received March 23rd, 1933.*)

In an earlier communication [Wiesner and Marshall, 1931] methods were described for the preparation of the gonadotropic principles of pregnancy urine in a solid form in which they could be stored indefinitely without loss of activity. One of these, that involving the use of phosphotungstic acid whereby the active principles were precipitated as phosphotungstates, has been shown to be satisfactory only for the preparation of extracts for use in certain laboratory animal experimentation; for this purpose the phosphotungstates were suspended in water and solution effected by the addition of alkali to p_H 7·0–7·5, such solutions being directly injected into mice which appear readily to tolerate relatively large amounts of phosphotungstates.

For clinical purposes, however, the phosphotungstic acid must be completely removed, a process which the author's recent work has proved to be difficult to accomplish satisfactorily (even as the barium salt) owing to the tendency of a part of the barium phosphotungstate to remain in colloidal solution. This process has therefore been abandoned.

The methods described by Dickens [1930] would no doubt be satisfactory for the preparation of clinical extracts, as would the benzoic adsorption method used by Funk and Zefirow [1932] and Katzman and Doisy [1932], but in the preparation of such extracts considerations of time and cost are of considerable importance if they are to find any extensive use. The method to be described here is really a simplification of an earlier alcohol precipitation process; the solid preparation usually contains 5–10 mouse units (luteinising) per mg. and is of sufficient purity for use in the preparation of clinical extracts containing 50 units per cc., which is the strength used for experiments now in progress. The benzoic acid adsorption method has been repeated according to the details given by Katzman and Doisy [1932] without any attempt being made to explore the conditions for maximum yield; the result was satisfactory, but the method does not appear to the author to offer any definite advantage over that to be described here. An attempt was made to use picric acid in obtaining solid ρ-factor preparations from urine, and one specimen was isolated with a potency of 20 mouse units (luteinising) per mg., a total yield of over 50,000 units being obtained from 15 litres of urine; the specimen was, however, not free from protein, and, on deproteinisation, a considerable loss was sustained.

Various attempts to separate a further quantity of active material from these protein residues have been made, but have not so far yielded results which justify the labour involved, although the problem is being pursued.

In some experiments on the stability of ρ-factors in various solutions some interesting results were obtained, notably where it was shown that no loss of activity occurred in solution in concentrated ammonia (sp. gr. 0·880) at 0° during 24 hours, an observation which formed the basis of one attempt to recover some active material from the protein residues previously mentioned. The stability of ρ-factors at p_H 7·0 in 0·5 % phenol solution at room temperature has also been investigated with a view to determining for how long a clinical extract should be used after receipt. It was also found that a very dilute solution kept frozen for a week showed no loss of activity [*cf.* Dickens, 1930].

Attempts to effect further purification of the active principles by precipitation from protein-free solutions with uranium acetate were unsuccessful owing to the fact that such precipitation was by no means quantitative, although such a method was used by Reiss and Haurowitz [1929] for crude hormone preparations.

In view of Gulland and Macrae's observation [1932] that palladous chloride is an effective precipitating agent for a wide variety of compounds including the purine bases, this reagent was tried; however, whilst the active principles were precipitated from untreated urine, no precipitation occurred when protein-free solutions were used, and the method was of no value for further purification.

The method of Katzman and Doisy was also inapplicable to protein-free solutions, under the conditions described by them and found to be satisfactory for untreated urine.

The potency of all samples used in clinical experiments together with any others used in this work was determined exactly as described by the author in a previous communication [1932]. The relative merits of the different criteria of a "mouse unit" will be dealt with in the discussion of the experimental results.

EXPERIMENTAL.

Preparation of clinical product by alcohol method. Urine, in batches of 10–12 litres adjusted to p_H 7·0, is evaporated under reduced pressure at 40° to 1/10–1/20 of its original volume and treated with an equal volume of industrial spirit (95 %). The resulting precipitate is discarded and the clear filtrate treated with more alcohol to a concentration of 75–80 %; after standing for some time, usually overnight, at 0° the bulk of the supernatant fluid can be decanted, and the precipitate is collected in the centrifuge and washed once with 90 % alcohol. It is suspended in water (60 cc.), and 15 cc. of 20 % sulphosalicylic acid are added together with 70 cc. of 95 % alcohol. The whole is then centrifuged and alcohol added to the clear supernatant fluid to a concentration of 75–80 %. The precipitate which collects after standing for some hours at 0° is removed by centrifuging and washed successively with 95 % alcohol, absolute alcohol and finally ether, after which it is dried in a current of air at room temperature. The material obtained in this way is collected from several batches and dissolved (or suspended) in 10 times its weight of water and submitted to one further alcohol precipitation, any precipitate obtained in 50 % alcohol solution being again discarded; the product, which should be completely soluble in water and protein-free, is then ready for standardisation in the usual way.

Precipitation of ρ-factors by alcohol at varying p_H. This was originally undertaken with the idea of obtaining a more active precipitate at some definite reaction or possibly of effecting a separation of the principles.

50 cc. of a stock solution of ρ-factors was prepared in 0·85 % saline containing 200–250 m.u. per cc. (p_H 5·55). Four groups were taken. (1) Original + 10 % H_2SO_4: p_H 0·94. (2) Original + 10 % acetic acid: p_H 3·5. (3) Original alone: p_H 5·55. (4) Original + NaOH: p_H 7·16. To each were added 4 volumes of absolute alcohol and the precipitates removed by centrifuging after standing for 1–2 hours at 0°. The supernatant fluid, after neutralisation and evaporation of the alcohol, was injected into immature mice in doses corresponding to 5·0 mg. (100–120 m.u.) of the original solid. Entirely negative results were obtained, showing that quantitative precipitation takes place within the range p_H 0·94–7·16 with 80 % alcohol; the precipitates were strongly active. At the same time it was observed that the precipitate formed at p_H 0·94 was much lighter in colour and entirely devoid of the stickiness which characterised the precipitates obtained at the other p_H values. This is of great value in facilitating manipulation in the final stages, and advantage has been taken of this fact in many of our recent preparations.

15 g. of crude material were suspended in 125 cc. of water (or preferably 0·85 % saline), and 10 % H_2SO_4 was added to p_H 0·9–0·95, about 25 cc. being required by an average sample but this quantity being subject to considerable variation. The precipitate formed on addition of an equal volume of alcohol was removed and the final product isolated as described earlier in the form of a very pale buff powder. There is an additional advantage in that the precipitate settles more readily and the bulk of the supernatant fluid can be decanted in a relatively short time without loss of active material. In this method all p_H values were determined by the use of the quinhydrone electrode.

Effect of ammonia (sp. gr. 0·880) on ρ-factors. A solution of 0·5 g. of ρ-factors (25 units per mg.) in 10 cc. of ammonia (sp. gr. 0·880) was allowed to stand at 0° for 18 hours. A small amount of insoluble matter was then removed in the centrifuge and the solution aerated (with addition of a drop of capryl alcohol) to remove as much ammonia as possible. The further precipitate produced on neutralisation with acetic acid to p_H 6·5 was removed by centrifuging, and the active principles were precipitated as usual by addition of alcohol. The yield was 0·4 g. of material, which was active in a dose of less than 0·05 mg., since 3 animals receiving this dose all showed many blood-points and corpora lutea (atretica). The above treatment therefore does not appear to have injured the active principles to any appreciable extent.

Action of ammonia (sp. gr. 0·880) on protein residues. 5·0 g. of protein residues (from ρ-factor preparations) in 50 cc. of ammonia were kept at 0° for 24 hours with occasional shaking. After addition of 150 cc. of water the mixture was kept cool whilst neutralising to p_H 6·5 with acetic acid. A further 25 cc. of 20 % sulphosalicylic acid was then added to remove unchanged protein, followed by an equal volume of alcohol. After centrifuging, the supernatant fluid was treated with alcohol to 80 % concentration, and the product was isolated as usual. A yield of 0·6 g. was obtained with an approximate luteinising unit of 1·0 mg. The results of the method do not therefore justify the labour involved.

A similar attempt to obtain active material from the protein residue by the use of 50 % alcohol containing 0·5 % of concentrated HCl at 0° was even less successful.

Loss of activity of ρ-factors in neutral solution. A phial containing a sterile solution of ρ-factors made up to contain about 50 m.u. per cc. (as used in clinical experiments) was kept at room temperature for 26 days. After this time doses equivalent to ½–3 units of the original gave no blood-points or corpora lutea in the test animals, but the vaginae were open and the uteri enlarged. A similar test after keeping 17 days showed that the solution had lost approximately 50 % of its activity. In one instance it was also observed that a dilute solution lost practically none of its activity when kept frozen for 7–10 days.

Adsorption of ρ-factors from urine by benzoic acid. 4 litres of urine were adjusted to p_H 4–5 with acetic acid and treated with a saturated solution of benzoic acid in acetone exactly as described by Katzman and Doisy [1932]. The precipitate, after dissolution of the contained benzoic acid in acetone, was extracted with 90 cc. of water and centrifuged, the supernatant fluid again being treated with 2½ volumes of acetone. The precipitate was collected as usual after standing overnight at 0°.

Yield = 0·12 g. with an activity of approximately 15 luteinising units per mg., which corresponds to 450 units per litre. It was not quite protein-free.

Attempt to adsorb ρ-factors on benzoic acid from protein-free solutions. 1 g. of ρ-factors (30 units per mg.) was dissolved in 200 cc. of water (p_H 5) and 20 cc. of a saturated solution of benzoic acid in acetone added with stirring. After standing overnight the precipitate was removed and treated as in the case of original urine. The yield was 0·06 g. of material of which 0·05 mg. failed to produce any effect in a group of mice. The filtrate from the benzoic acid precipitate was treated with 2–3 cc. of saturated KCl and 4 volumes of alcohol. A yield of 0·78 g. was obtained of material which had an activity of 40 units per mg. The active principles therefore appear to pass quantitatively into the benzoic acid filtrate under these conditions.

Preparation of clinical extracts. The solid preparations used for this purpose have usually contained about 5 luteinising units per mg., and 0·1 g. has been dissolved for every 10 cc. of solution required. In the first instance the solution is almost invariably acid through the presence of traces of sulphosalicylic acid, which are quite harmless when neutralised; sodium hydroxide is therefore used to bring the solution to p_H 7 or thereabouts (B.D.H. universal indicator), after which 0·5 % of phenol is dissolved in it. It is then filtered through a Seitz bacterial filter into sterilised apparatus, whence it is run into sterile ampoules. A stringent sterility test, using bullock's heart medium, is always carried out by the courtesy of the Royal College of Physicians Laboratory, Edinburgh, before any extract is issued.

Controlled precipitation of ρ-factors by phosphotungstic acid. To 1 litre of urine (filtered, p_H 7·3) were added 50 cc. of 20 % phosphotungstic acid (adjusted to p_H 6·1 with NaOH). No precipitate occurred, but on adding 10 % H_2SO_4 gradually the solution became definitely opalescent at p_H 4·5, and a flocculent precipitate began to settle at p_H 3·5. After standing for 1 hour in the cold the bulk of the supernatant fluid was decanted, the precipitate collected and washed with 1 % phosphotungstic acid at p_H 3·5. The precipitate was suspended in water and adjusted to p_H 7·0–7·5 with sodium hydroxide, any insoluble matter being removed. The total volume was made up to 500 cc., and 0·5 cc. of this gave a very strong positive ovarian reaction when injected in the usual way, *i.e.* the equivalent of 1 cc. of original urine contained 1 luteinising unit. A further precipitate obtained by continued addition of 10 % H_2SO_4 to the filtrate proved very toxic to the animals, but from the results obtained with the precipitate at p_H 3·5 the precipitation at that reaction must be fairly quantitative. This method was repeated several times with similar results, but in each case the removal of the phosphotungstic acid from the active precipitate was attended with such a large loss that the method was abandoned for the preparation of clinical extracts. It is interesting to note that, although ρ-factors can be precipitated from protein-free solutions by phosphotungstic acid, precipitation can only be effected at a p_H considerably lower than 3·5, the reaction at which precipitation is almost quantitative from untreated urine.

Discussion.

One of the most striking points which one encounters in the precipitation reactions of the gonadotropic hormones is the difference in the results obtained according as one uses original untreated urine or a product already partially purified. In a previous communication [Marshall, 1932] it was shown that the precipitability by alcohol was dependent to a considerable extent on the inorganic salt content of the solution, and that where this was almost negligible the further addition of ether was required before precipitation could be effected, and Dickens [1930] recorded a case in which he was unable to precipitate the product even by this means, although in this latter instance the solution was probably relatively dilute. In the present paper it has been shown that the p_H of the solution has little effect on the quantitative precipitation of ρ-factors by 80 % alcohol provided that sufficient inorganic salts are present, except in so far as a less gummy precipitate is obtained at more acid reactions. In adsorption experiments with benzoic acid, the work of Katzman and Doisy on untreated urine has been confirmed, whilst no adsorption whatever occurred under similar conditions from protein-free extracts. It therefore appears that the presence of protein and protein-like substances other than the active principles exert profoundly modifying influences on the chemical and physical behaviour of ρ-factors. Whichever of the published methods for obtaining potent gonadotropic extracts be adopted, a very considerable loss occurs during the removal of protein material at some stage of the operations; this is evidently due to powerful adsorption under the conditions used, since alkali is necessary to extract any further quantity of active material, a process which unfortunately also dissolves some of the protein. In the near future it is hoped to make some attempt to obtain increased yields by lessening the waste at present entailed in deproteinisation.

Turning to the question of standardisation of gonadotropic hormones, it is at once evident both that careful standardisation is necessary for extracts used in any clinical experiments, and also that no entirely satisfactory method of accomplishing this has yet been worked out. In the first place it is obvious that a complete standardisation is a matter of considerably greater difficulty than is the case with oestrin standardisation, in which, although one may be dealing, in any given preparation, with a mixture of trihydroxy- and ketohydroxy-oestrin, apparently only one effect need be considered, namely the production of cornification of the vaginal epithelium of the ovariectomised mouse or rat. With the gonadotropic hormones it is necessary to consider the growth and maturation of immature follicles, the production of haemorrhagic follicles, and the formation of corpora lutea (or atretica), together with obvious ovarian hypertrophy and other effects such as vaginal canalisation and cornification of the epithelium. It was shown in Part I of this series [Wiesner and Marshall, 1931] that opening of the vagina in the immature mouse along with cornification might occur with a small dose of a given preparation, whilst with a much larger dose frequently neither of these effects occurred, although in the latter case ovarian hypertrophy was many times greater than in the former. This observation was then given as further support for the existence of two distinct factors, a hypothesis advanced by several workers, although it was recognised that this fact by itself could prove nothing since the same substance may have opposite effects in small and large doses. The extent of the antagonism of these two factors is still unknown and cannot possibly be elucidated until final proof is forthcoming of their separate existence. A further complicating factor is the observation by the author, in the course of many hundreds of experiments, that the number and size of blood-points is many times greater with the larger doses, and that corpora atretica may occur without the formation of any blood-points. The evidence is, as yet, not sufficiently correlated to warrant any suggestion of the existence of a third factor, which, however, is not impossible, although it has previously been assumed that ρ_1 (Prolan A) is responsible both for maturation of the follicles and for the formation of blood-points. Although Zondek [1930] has obtained a product from the urine of patients with genital carcinoma which apparently only gives an oestrogenic effect, and Claus [1931] reported the separation of the oestrogenic and luteinising factors from dried pituitary preparations, the existence of more than one factor is still without final proof since the latter work has not been repeated and no claim of a separation of the two from products of pregnancy urine has yet been made. It is necessary to direct further work to this very important aspect of the problem.

Katzman and Doisy [1932] have stated that they "believe that it is correct to use the characteristic reaction, *i.e.* stimulation of follicle ripening, as a measure of potency and that this reaction lends itself more easily to a quantitative determination." The author believes that this view is quite unjustified in the present state of our knowledge and particularly in relation to the clinical experiments now being carried out in Edinburgh. Only if one were dealing with a single factor could this statement be literally true. Until the quantitative antagonism of ρ_1 and ρ_2 has been worked out, we can have no absolutely accurate knowledge of the relationship between an "oestrogenic" and a "luteinising" unit, in fact it is highly probable that the ratio of the two is variable for different specimens.

For the purposes of approximate standardisation of the clinical extracts used it was considered better to determine the "luteinising" unit, since it was this effect which it was desired to produce in the treatment of dysmenorrhoea and certain other disorders. The actual procedure has been referred to previously.

The discovery that no loss of activity occurs in solution in ammonia (sp. gr. 0·880) in 24 hours at 0° is interesting as indicating a greater stability under these conditions than one might have been led to suppose.

It will be noticed that after removal of proteins the active principles are left for 18 hours at 0° in 75–80 % alcoholic solution containing approximately 1 % of sulphosalicylic acid. Dickens [1930] has suggested that the loss of activity which he observed in solutions kept at 0° for a month or thereabouts was due to the slight acidity of the solution; this may very well be the case although the author is of the opinion that, at least at room temperature, such inactivation would proceed slowly even at neutrality. Although the methods described have yielded satisfactory extracts for the purpose desired, it is proposed, and it seems desirable, to determine whether appreciable inactivation occurs by the use of sulphosalicylic acid under the conditions indicated above.

SUMMARY.

1. Details are given for obtaining a gonadotropic hormone preparation from pregnancy urine suitable for use in clinical extracts. Solution is effected in 0·5 % phenol.

2. The stability of such sterile solutions at room temperature has been examined, and they have been found to lose approximately 50 % of their activity in the course of 17 days and 75 % or more in 26 days. Little activity is lost when extracts are kept frozen for 7–10 days.

3. ρ-Factors can be kept at 0° in solution in ammonia (sp. gr. 0·880) for 24 hours without appreciable loss of activity. An attempt, based on this finding, to obtain further quantities of ρ-factors from the protein residues proved uneconomic.

4. ρ-Factors cannot be adsorbed on benzoic acid at p_H 5 from partially purified protein- and phosphate-free preparations.

5. Palladous chloride cannot be used for further purification of protein-free specimens.

6. Criteria of different methods of standardisation are discussed, and it is shown that a completely satisfactory method must of necessity be more difficult to obtain than in the case of oestrin.

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LXXXIV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXIX. 2:5-DIHYDROXYBENZOIC ACID (GENTISIC ACID) A NEW PRODUCT OF THE METABOLISM OF GLUCOSE BY *PENICILLIUM GRISEO-FULVUM* DIERCKX.

By HAROLD RAISTRICK and PAUL SIMONART.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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In Part XIX of this series [Anslow and Raistrick, 1931] it was shown that when *Penicillium griseo-fulvum* Dierckx is grown at 24° on Czapek-Dox medium containing glucose (5 %) as sole source of carbon, the metabolism solution, after some days, gives an intense purple colour with ferric chloride. The substance responsible for this coloration was isolated and identified as 2-hydroxy-6-methylbenzoic acid (6-methylsalicylic acid), but at that time no search was made for other phenolic metabolic products, since the above acid was undoubtedly the chief phenolic product formed under these conditions and was produced in good yield (2·42 % of the glucose metabolised).

This work has now been continued, and in order to determine the optimum conditions for the production of phenolic substances by this mould small scale experiments (in test-tubes) have been carried out at 20, 24, 28, 30 and 35°. It has been found that the optimum temperature for sugar utilisation and production of phenols, as measured by the bromine absorption method of Koppeschaar [1876], is 30°. At this temperature also, sporing is much heavier than at 24°. The mould has also been grown on various modified Czapek-Dox media, the temperature of incubation being always 30°. It has been found that when the glucose content of the medium was 8 % the bromine absorption value, in mg. Br per cc. of metabolism solution after 40 days' incubation, was much higher than was obtained with the corresponding metabolism solutions when the media contained initially only 4, 5 or 6 % glucose, the actual value being 6 mg. per cc. as compared with 2·5, 2·7 and 3·8 mg. respectively. The sequence of colours with ferric chloride was also rather different. For example, after 40 days' incubation, the colour obtained with 8 % glucose was no longer purple but dark blue, changing with excess $FeCl_3$ to dark brown, whereas the corrresponding colour for media containing less glucose was still purple. It was therefore evident that 6-methylsalicylic acid was not the only phenolic metabolic product, and in order to arrive at still better conditions for the production of the second phenolic substance experiments were carried out with media containing 8 % glucose, but having different glucose-sodium nitrate ratios. Although, after 40 days, the bromine absorption values were the same for media with different ratios of glucose to sodium nitrate, the ferric chloride colours were different, *e.g.* when the ratio was 25 : 1 (*i.e.* the ratio in the usual 5 % Czapek-Dox glucose medium), the colour was purple, though when the ratios were 16 : 1 and 32 : 1 the colours were dark brown and dark blue respectively.

The medium chosen for large scale experiments contained therefore 8 % of glucose and 0·25 % of sodium nitrate (ratio = 32 : 1).

Isolation of the new metabolic product responsible for the blue colour with ferric chloride.

A quantity of Czapek-Dox medium, modified as indicated above, was made up, having the following composition: glucose, 80 g.; $NaNO_3$, 2·5 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4, 7H_2O$, 0·5 g.; $FeSO_4, 7H_2O$, 0·02 g.; distilled water, 1000 cc. One hundred and ten 1-litre conical flasks, each containing 350 cc. of medium, were sterilised by steam on three consecutive days. They were sown with a suspension of spores (from a 10-day pure culture of *P. griseo-fulvum* Dierckx grown on beer wort agar at 30°) and incubated at 30° for 45 days. An average sample of the filtered metabolism solution contained 1·12 % glucose, the bromine absorption corresponded to 3·7 mg. per cc., and ferric chloride gave a dark brown colour. The metabolism solution was concentrated to 1 litre *in vacuo* below 50°. From the precipitate which separated during evaporation a considerable quantity of crude mannitol was isolated (120 g.). After crystallisation from aqueous alcohol, it melted at 163–164°, and the M.P. was not depressed by admixture with an authentic specimen of mannitol. Further, the purified substance was optically inactive, but when borax (3 g.) was added to a solution of the substance (0·270 g.) in water (25 cc.) and the whole diluted to 50 cc., the observed rotation was + 0·4° in a two dm. tube, using the mercury yellow line $\lambda = 5790$. This corresponds exactly with the value calculated from the tables given by Raistrick and Young [1931] and proved conclusively that the metabolic product was mannitol. The filtrate from the mannitol was made acid to Congo red with dilute sulphuric acid and extracted with an equal volume of chloroform until the extract gave no colour with ferric chloride, three extractions being necessary. In this way all the 6-methylsalicylic acid was removed, since it is moderately soluble in chloroform, while the new metabolic product is almost insoluble in this solvent in the cold. The liquid was then repeatedly extracted with an equal volume of ether until the extract no longer gave a blue colour with ferric chloride. Evaporation of the ether yielded a brown semi-crystalline product which was submitted to a long series of fractional crystallisations from chloroform-light petroleum. Two crystalline acids were isolated, the less soluble subliming at about 200° and melting at 272–274° in a closed M.P. tube; the more soluble having M.P. about 195°.

Identification of the less soluble acid as fumaric acid.

This product gave no colour with ferric chloride and was identified as fumaric acid, a mixed melting-point with authentic fumaric acid (M.P. 286°) being about 280° in a closed M.P. tube. Micro-analysis (Schoeller): C, 41·50, 41·62; H, 3·53, 3·52 %. $C_4H_4O_4$ requires C, 41·38; H, 3·48 %.

0·0100 g. required 8·8 cc. $N/50$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 57, the theoretical value for $C_4H_4O_4$ titrating as a dibasic acid being 58.

Purification and properties of the second phenolic metabolic product.

The second and more soluble metabolic acid mentioned above gave a deep blue colour with ferric chloride and was purified by sublimation followed by crystallisation from ethyl acetate-light petroleum or from toluene. It formed colourless needles, M.P. 197–198°, but the melting-point can with difficulty be raised to 202° after repeated crystallisations from ethyl acetate-light petroleum, involving much loss of material. Micro-analysis (Schoeller): C, 54·85, 54·91; H, 4·04, 4·09 %; Mol. wt., 139, 158. $C_7H_6O_4$ requires: C, 54·52; H, 3·92 %; Mol. wt., 154.

0·1910 g. required 12·3 cc. *N*/10 NaOH for neutralisation to phenolphthalein corresponding to an equivalent of 155, the theoretical for $C_7H_6O_4$ titrating as a monobasic acid being 154.

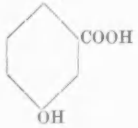
The new metabolic product is readily soluble in the cold in water, alcohol, ether and ethyl acetate. It is sparingly soluble even in hot chloroform, moderately soluble in hot toluene and almost insoluble in light petroleum.

To obtain more of the new metabolic product 330 1-litre flasks containing the same quantity of medium were sown as mentioned above. The time of incubation was extended to 65–70 days. After that period the average glucose content was 0·6 %, the average bromine absorption corresponded to 4 mg. per cc. and the metabolism solution now gave a dark blue colour with ferric chloride. The metabolism solution was concentrated *in vacuo* to 6 litres and treated exactly as described above. The chloroform extracts yielded a brown crystalline material (crude 2-hydroxy-6-methylbenzoic acid; yield 61·2 g., corresponding to 0·72 % of the glucose metabolised). The ether extracts after drying over anhydrous Na_2SO_4 yielded on evaporation a dark brown semi-crystalline material (114·1 g., *i.e.* 1·34 % of the weight of glucose metabolised). This product was boiled several times with toluene, which does not dissolve the dark brown impurity, and the new metabolic product crystallised from the toluene extracts on cooling. The crude crystalline product (11 g. representing 0·12 % of the weight of glucose metabolised) was recrystallised several times from toluene (charcoal).

The residues obtained by Anslow and Raistrick [1931] by evaporation of the mother-liquors from the crystallisation of 6-methylsalicylic acid were also examined and both fumaric acid and the new phenolic product $C_7H_6O_4$, M.P. 197–198°, were isolated in small amounts.

Identification of the new metabolic product as gentisic acid (2 : 5-dihydroxybenzoic acid).

The ferric chloride reaction (deep blue, slowly fading to brown) suggests that the substance is a derivative of quinol, and its empirical formula, $C_7H_6O_4$, and the fact that it titrates as a monobasic acid show clearly that it is probably quinolcarboxylic acid (2 : 5-dihydroxybenzoic acid, gentisic acid (I))



(I)

A mixed melting-point with a specimen of synthetic gentisic acid, M.P. 197–198°, prepared from quinol and KHCO$_3$ by the method of Brunner [1907] showed no depression, and the identity of the new metabolic product with gentisic acid was settled beyond all doubt by the preparation of the following derivatives, all of which were identical (by M.P. and mixed M.P.) with the corresponding derivatives prepared by parallel experiments carried out on synthetic gentisic acid: (*a*) the methyl ester, M.P. 85–86°, (*b*) the monomethyl ether (5-methoxy-2-hydroxybenzoic acid), M.P. 141–142°, prepared by methylation of gentisic acid with diazomethane and hydrolysis of the resulting phenolic ester with hot alkali, (*c*) the diacetyl derivative, M.P. 119–121°. Finally, (*d*) quinol itself was obtained by heating the acid above its melting-point and resublimation of the crystalline sublimate.

Preparation of derivatives of the new metabolic product (gentisic acid).

(*a*) *Methyl ester* [*cf.* Graebe and Martz, 1905]. A solution of the pure acid (0·5 g.) in methyl alcohol (10 cc.) containing 3 % HCl was boiled under reflux for 3 hours, diluted with water, neutralised by addition of NaHCO$_3$ and extracted with ether. The residue left after evaporation of the dried ether extract was crystallised from chloroform-light petroleum to yield colourless prisms, M.P. 85–86°, which gave a blue colour with ferric chloride. A mixture of the above product with authentic methyl gentisate (M.P. 85–86°) also melted at 85–86°.

Micro-analysis (Schoeller): C, 57·34, 57·15 %; H, 4·86, 4·83 %; OCH$_3$, 18·13, 18·20 %; mol. wt. (in camphor), 178, 186. C$_7$H$_5$O$_3$(OCH$_3$) requires C, 57·12; H, 4·79 %; OCH$_3$, 18·45 %; mol. wt., 168.

(*b*) *Methylation by diazomethane and hydrolysis of the methylation product; formation of 5-methoxy-2-hydroxybenzoic acid.* To the pure metabolic product (0·5 g.) was added ethereal diazomethane prepared from nitrosomethylurethane (10 cc.). After 24 hours the ether was removed, and the pale brown oily residue (0·60 g.) was found still to give a blue colour with ferric chloride. As was anticipated, the phenolic hydroxyl group *ortho* to COOH had escaped methylation, and the product was doubtless essentially the methyl ester of 5-methoxy-2-hydroxybenzoic acid. The crude ester was hydrolysed by boiling for 1 hour with a mixture of alcohol (20 cc.) and *N* NaOH (20 cc.). The resulting solution was made acid to Congo red by addition of dilute sulphuric acid and extracted with ether. The dark-coloured residue remaining after evaporation of the dried ether extract was crystallised from water (charcoal) to yield light, colourless needles, M.P. 141–142°, an aqueous solution of which gave a deep blue colour with ferric chloride and reacted acid to litmus.

Micro-analysis (Schoeller): C, 57·03, 57·11; H, 4·81, 4·81; OCH$_3$, 18·72, 18·17 %. C$_7$H$_5$O$_3$(OCH$_3$) requires C, 57·12; H, 4·79; OCH$_3$, 18·45 %.

The melting-point of 5-methoxy-2-hydroxybenzoic acid is variously given in the literature as 141, 142 and 143·5°. When prepared from synthetic gentisic acid by the above method it melted at 141–142° and a mixture of the synthetic compound with the above monomethyl ether of the metabolic product also melted at 141–142°.

(*c*) *Diacetyl derivative* (2:5-diacetoxybenzoic acid). A solution of the pure metabolic product (0·5 g.) in pyridine (5 cc.) and acetic anhydride (5 cc.) was kept at 37° for 2 days. The dark-coloured liquid was then poured into water (100 cc.). The solution was acidified with dilute sulphuric acid and extracted with ether. The residue left after evaporation of the ether was crystallised from water and yielded colourless crystals, M.P. 116–117°, which gave no colour with ferric chloride. The yield was very small.

Micro-analysis (Schoeller): C, 55·00; H, 4·30 %. C$_{11}$H$_{10}$O$_6$ requires C, 55·44; H, 4·23 %.

Synthetic 2:5-diacetoxybenzoic acid was prepared by boiling a solution of gentisic acid in excess of acetic anhydride together with some anhydrous sodium acetate for 3 hours, according to the method of Hemmelmayr [1909]. It formed colourless crystals, M.P. 118–120°. A mixture of this synthetic compound with the above diacetyl derivative of the metabolic product melted at 116–119°.

A sample of the diacetyl derivative of the metabolic product was also prepared by boiling 0·5 g. of the latter with anhydrous sodium acetate (0·5 g.) and acetic anhydride (2 cc.) for 30 minutes.

The reaction mixture was diluted with water (100 cc.), acidified with dilute sulphuric acid and the precipitate collected and fractionally crystallised from water. A crystalline product was obtained, which had M.P. 119–121°, gave no colour with $FeCl_3$ and was identical with synthetic 2:5-diacetoxybenzoic acid.

Micro-analysis (Schoeller): C, 55·42, 55·61; H, 4·28, 4·27 %; mol. wt. (in camphor), 213, 207. $C_{11}H_{10}O_6$ requires C, 55·44; H, 4·23 %; mol. wt., 238.

(*d*) *Decomposition by heat with formation of quinol.* The pure metabolic product (a few mg.) was heated in a small test-tube at 250–275°. The sublimate which collected on the cool part of the tube consisted of colourless plates and needles. The experiment was repeated several times, and the combined sublimates were resublimed at 150°. The final product gave a faint and transient blue colour with ferric chloride, reduced Fehling's solution on heating, and melted at 166–167°. A mixture of this substance with a specimen of authentic quinol (M.P. 168–169°) melted at 166–169°.

Micro-analysis (Schoeller): C, 64·74; H, 5·35 %; mol. wt. (in camphor), 128, 120. $C_6H_6O_2$ requires C, 65·42; H, 5·49 %; mol. wt., 110.

DISCUSSION.

It is thus seen that from the metabolism solution obtained when *P. griseo-fulvum* Dierckx is grown under the conditions described in a medium containing glucose as sole source of carbon four metabolic products have been isolated, *viz.* 6-methylsalicylic acid, gentisic acid, fumaric acid and mannitol. Since the sequence of ferric chloride colours given by the metabolism solution during the growth of the mould indicates that 6-methylsalicylic acid is the first phenolic product to appear, it seems quite possible that gentisic acid is an oxidation product of the above acid. Salicylic acid itself can be oxidised to gentisic acid by potassium persulphate [Graebe and Martz, 1905], and if the mould does indeed oxidise 6-methylsalicylic acid to gentisic acid, a hydroxyl group must be introduced, the methyl group in the former must be oxidised to carboxyl, and one carboxyl group must be eliminated as CO_2. It must be admitted, however, that we have not been able to isolate from the metabolism solution any of the possible intermediates in this oxidation.

This is the first recorded instance of the isolation of free gentisic acid from any natural source. It has, however, been obtained by potash fusion from the colouring matter gentisin (the methyl ether of 1 : 3 : 7-trihydroxyxanthone) extracted from the gentian root (*Gentiana lutea*).

There are a few instances in the literature of the production of fumaric acid by fungi, *i.e.* by *Rhizopus nigricans* [Ehrlich, 1911; Butkewitsch and Fedoroff, 1929], and by *Aspergillus fumaricus* [Wehmer, 1918; 1928], although, as far as we are aware, it has not previously been detected among the metabolic products of species of *Penicillium*.

SUMMARY.

The metabolism of glucose by *Penicillium griseo-fulvum* Dierckx has been further examined. A hitherto undescribed mould metabolic product, gentisic acid (2 : 5-dihydroxybenzoic acid), was isolated together with the previously described 2-hydroxy-6-methylbenzoic acid, and an attempt has been made to determine the optimum conditions for the production of gentisic acid.

In addition, mannitol and fumaric acid were isolated, thus affording the first example of the production of fumaric acid by a species of *Penicillium*.

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LXXXV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXX. THE MOLECULAR CONSTITUTION OF THE METABOLIC PRODUCTS OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX AND RELATED SPECIES.

I. THE ACIDS $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ AND $C_{10}H_{10}O_7$.

BY ALBERT EDWARD OXFORD AND HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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IN Part XXIV of this series [Clutterbuck, Oxford, Raistrick and Smith, 1932] it was shown that out of 15 species in the *P. brevi-compactum* series submitted to examination, 14 metabolised glucose into a mixture of phenolic acids, *viz.* mycophenolic acid $C_{17}H_{20}O_6$ (formed by 12 species only), and the new metabolic products $C_8H_6O_6$, $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, the last two being detected in every instance and the first two being probably formed by all 14 species although not always detectable with certainty on account of the smallness of the respective yields. The question of the molecular constitution of mycophenolic acid is dealt with in the accompanying paper [Clutterbuck and Raistrick, 1933] and the substance $C_8H_6O_6$ has been shown to be 3:5-dihydroxyphthalic acid [Oxford and Raistrick, 1932]. The present paper deals with the elucidation of the molecular constitutions of the three remaining substances, the acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$.

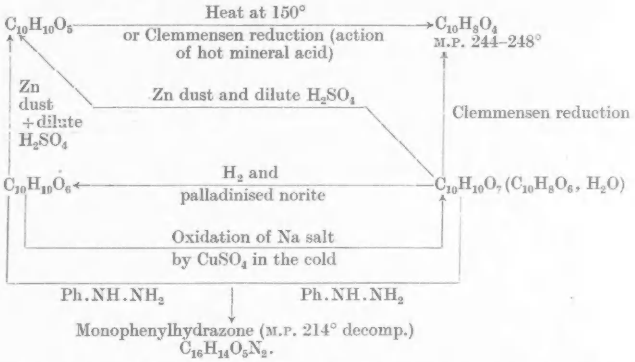
It will be convenient first to summarise the more obvious properties of these compounds as recorded in Part XXIV. All three are optically inactive. All give intense reddish-purple colorations with ferric chloride. $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_6$ (but not $C_{10}H_{10}O_7$) give solutions in cold concentrated H_2SO_4 which fluoresce blue in ultra-violet light, while $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ (but not $C_{10}H_{10}O_5$) give very dark colours when gently warmed with this reagent. All titrate more nearly as dibasic than as monobasic acids, but the end-point is very indefinite since the substances give brownish-red solutions with excess of alkali.

All three melt with elimination of water and some CO_2, $C_{10}H_{10}O_5$ at 145–153° losing nearly one molecule of water (and also 0·1 mol. CO_2) to give as chief product an entirely new substance $C_{10}H_8O_4$; $C_{10}H_{10}O_6$ at about 190–200° with elimination of water to give a crude product not yet examined further, also melting at 190–200° but without effervescence; $C_{10}H_{10}O_7$ at 125–135° losing $1H_2O$ (and practically no CO_2) to yield a syrup which reverts to the original substance when crystallised from solvents containing only traces of water.

$C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ are ketones giving immediate yellow precipitates with Brady's 2:4-dinitrophenylhydrazine reagent, while $C_{10}H_{10}O_6$ gives a precipitate with this reagent only on long standing. None of the compounds restores the colour to Schiff's reagent or has any other aldehydic properties.

It was soon established that the loss of water on heating the substance $C_{10}H_{10}O_7$ does not result in any fundamental constitutional change, and hence for all practical purposes the formula may be written $C_{10}H_8O_6, H_2O$. Thus the mono-2:4-dinitrophenylhydrazone (precipitated by the addition of Brady's reagent) when recrystallised and dried at 50° *in vacuo* analysed correctly for the dinitrophenylhydrazone of $C_{10}H_8O_6$. It is significant, however, that this derivative takes up $2H_2O$ when exposed to moist air. More convincing still, the catalytic reduction of $C_{10}H_{10}O_7$ yielded $C_{10}H_{10}O_6$ identical with the metabolic product of this formula, and, since oxygen atoms are not usually eliminated and replaced by hydrogen under the conditions employed, it follows that the original compound must have lost the elements of water under the mild conditions of the reduction.

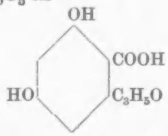
The obvious working hypothesis that all three substances are closely related, naturally led to attempts to effect inter-conversions *in vitro*, and these attempts met with considerable success. For example the sodium salt of $C_{10}H_{10}O_6$ was found to reduce copper sulphate in the cold (although neither $C_{10}H_{10}O_5$ nor $C_{10}H_{10}O_7$ reduces Fehling's solution even on boiling) some $C_{10}H_{10}O_7$ being formed. Further, as mentioned above, the catalytic reduction of $C_{10}H_{10}O_7$ (*i.e.* $C_{10}H_8O_6, H_2O$) yielded $C_{10}H_{10}O_6$, while $C_{10}H_{10}O_7$ and $C_{10}H_{10}O_6$ both gave rise to $C_{10}H_{10}O_5$ when reduced by zinc dust and dilute sulphuric acid at 80–100°. Reduction by Clemmensen's method rather unexpectedly converted both $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_7$ into $C_{10}H_8O_4$, the dehydration product of $C_{10}H_{10}O_5$. Finally it may be mentioned that both $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ ($C_{10}H_8O_6, H_2O$) yield the same monophenylhydrazone $C_{16}H_{14}O_5N_2$. Thus $C_{10}H_{10}O_7$ ($C_{10}H_8O_6, H_2O$) is a ketone and $C_{10}H_{10}O_6$ is oxidisable to the same ketone by phenylhydrazine. These relationships are summarised in the following scheme:


$C_{10}H_{10}O_5$
Heat at 150°
or Clemmensen reduction (action of hot mineral acid)
$C_{10}H_8O_4$
M.P. 244–248°
Zn dust + dilute H_2SO_4
Zn dust and dilute H_2SO_4
Clemmensen reduction
H_2 and
palladinised norite
$C_{10}H_{10}O_6$
$C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O)
Oxidation of Na salt
by $CuSO_4$ in the cold
Ph.NH.NH$_2$
Ph.NH.NH$_2$
Monophenylhydrazone (M.P. 214° decomp.)
$C_{16}H_{14}O_5N_2$.

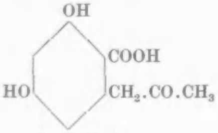
Thus it is clear that all three metabolic products contain the same carbon skeleton and differ only as regards the state of oxidation of the C atoms in the side-chains attached to the benzene ring. Hence, since the oxidation of $C_{10}H_{10}O_7$ ($C_{10}H_8O_6, H_2O$) by alkaline hydrogen peroxide was found to yield some 3:5-dihydroxyphthalic acid, each of the three C_{10} compounds must be a resorcinol derivative with two side-chains *ortho* to each other. This was conclusively proved by the isolation of 3:5-dimethoxyphthalic acid in good yield by the oxidation of the fully methylated derivative of $C_{10}H_8O_4$ by alkaline $KMnO_4$ in the cold.

Since all the oxygen atoms in $C_{10}H_{10}O_5$ can be accounted for (two in —COOH, two as phenolic OH groups and one in a carbonyl group), the possibility of a second hetero-ring attached to the benzene nucleus is ruled out, and the only possibilities for the complete carbon skeleton are $C_6\left\langle\begin{matrix}C\\C_3\end{matrix}\right.$ and $C_6\left\langle\begin{matrix}C_2\\C_2\end{matrix}\right.$. The second possibility can be dismissed at once since there is conclusive evidence that the carboxyl group in $C_{10}H_{10}O_5$ is directly attached to the benzene ring. The dimethyl ether of $C_{10}H_{10}O_5$, *i.e.* $C_{12}H_{14}O_5$, loses both H_2O (0·9 equivalent) and CO_2 (0·1 equivalent) when heated at 180°. The product when hydrolysed by hot alkali yields chiefly the original $C_{12}H_{14}O_5$ together with a relatively small amount of a neutral oily ketone, which can be extracted from the alkaline hydrolysis liquid by ether. This ketone when oxidised by alkaline permanganate in the cold yields not 3:5-dimethoxyphthalic acid but 3:5-dimethoxybenzoic acid identical with an authentic specimen obtained by the oxidation of the dimethyl ether of orcinol.

Hence we may write $C_{10}H_{10}O_5$ as

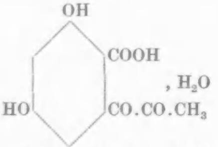


and since it is a ketone the side-chain $—C_3H_5O$ may be either $—CH_2.CO.CH_3$ or $—CO.CH_2.CH_3$. There is little doubt that the former formula is correct since the dimethyl ether of $C_{10}H_{10}O_5$ (like benzylmethylketone, unlike propiophenone) yields iodoform when oxidised by hypoiodite under the conditions prescribed by Birkinshaw and Raistrick [1931] for the estimation of kojic acid. A control experiment with 3:5-dimethoxyphthalic acid showed that the benzene ring in fully methylated resorcinol derivatives is quite stable under these conditions. Hence $C_{10}H_{10}O_5$ must have formula I, and $C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O) can therefore be represented only by formula II, while $C_{10}H_{10}O_6$ must have formula III or a tautomeric form with side-chain $—CO.CHOH.CH_3$, $—C(OH){=}C(OH)CH_3$, $—\underbrace{CH—C}_{O}(OH)CH_3$ or $—\underbrace{C(OH)—CH}_{O}.CH_3$, or an equilibrium mixture of two or more of these possible tautomerides.

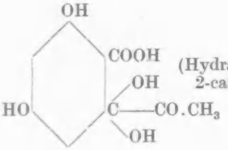


(3:5-dihydroxy-2-carboxybenzylmethylketone)

I. $C_{10}H_{10}O_5$

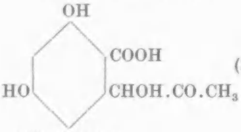


, H_2O or



(Hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone)

II. $C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O)

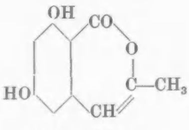


(3:5-dihydroxy-2-carboxyphenylacetylcarbinol)

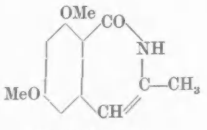
III. $C_{10}H_{10}O_6$

It remains to be shown that these constitutional formulae are in full agreement with all the known properties of the three C_{10} metabolic products. As will be mentioned later there are, however, one or two highly interesting exceptions in the case of II ($C_{10}H_{10}O_7$).

I. $C_{10}H_{10}O_5$. The metabolic product $C_{10}H_{10}O_5$ resembles orsellinic acid (3:5-dihydroxy-2-carboxytoluene) in many respects, *e.g.* in its ferric chloride reaction, its dibasic nature and its ability to give coloured solutions with excess of alkali. The above constitutional formula shows that $C_{10}H_{10}O_5$ is very closely related to orsellinic acid, being in fact its *C*-acetyl derivative. The fact that $C_{10}H_{10}O_5$ readily loses water on heating or by the action of hot mineral acids to yield the non-ketonic $C_{10}H_8O_4$—a feeble (monobasic) acid not very readily hydrolysed back again to $C_{10}H_{10}O_5$ by alkali—also finds a simple explanation. According to Gottlieb [1899], *o*-carboxybenzylmethylketone is readily converted into 3-methyl*iso*coumarin by the action of hot dilute mineral acids, and on this analogy, $C_{10}H_8O_4$ must be 6:8-dihydroxy-3-methyl*iso*coumarin (IV).



IV. $C_{10}H_8O_4$

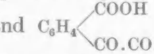


V. $C_{12}H_{13}O_3N$

$C_{10}H_8O_4$ should therefore contain two phenolic OH groups like $C_{10}H_{10}O_5$, and there is ample evidence to show that neither of the phenolic OH groups in the latter is concerned in the elimination of H_2O. For example, $C_{10}H_8O_4$ yields a neutral diacetyl derivative which can also be obtained by the acetylation of $C_{10}H_{10}O_5$. $C_{10}H_8O_4$ also yields a neutral non-phenolic dimethyl derivative when methylated by CH_3I and K_2CO_3 in acetone. This derivative can also be obtained by methylation of the methyl ester of the monomethyl ether of $C_{10}H_{10}O_5$ (formed when $C_{10}H_{10}O_5$ is methylated by diazomethane) by the same method. It yields 3:5-dimethoxyphthalic acid on oxidation, and, when hydrolysed by hot alkali, gives a non-phenolic keto-acid $C_{12}H_{14}O_5$ containing two methoxyl groups, evidently 3:5-dimethoxy-2-carboxybenzylmethylketone. The fact that diazomethane methylates only one of the phenolic OH groups of $C_{10}H_{10}O_5$ is doubtless to be explained by the presence of COOH *ortho* to one of the OH groups. In $C_{10}H_8O_4$ this same OH group is still in the *ortho* position to a carbonyl group (hence the feebly acidic properties of $C_{10}H_8O_4$) and it was found also that the methylation of $C_{10}H_8O_4$ even by a tenfold excess of diazomethane yielded a mixture of a feebly acidic monomethyl ether (25 %) together with the dimethyl ether (75 %).

A well known property of *iso*coumarins is that they can be converted into *iso*carbostyril derivatives by heating in a sealed tube with alcoholic ammonia, the —CO—O— being changed into —CO—NH—. The dimethyl ether of $C_{10}H_8O_4$ undergoes a similar transformation, the product $C_{12}H_{13}O_3N$ (V) having the expected properties, being insoluble in alkali and not very readily soluble in dilute HCl.

II. $C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O). The molecular constitution suggested for $C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O) presents certain difficulties. Thus $C_{10}H_8O_6$ is represented as an α-diketone and its hydrated form $C_{10}H_{10}O_7$ does in fact condense with *o*-phenylenediamine to give a derivative analysing correctly for the expected quinoxaline, but this crystalline hydrated form, $C_{10}H_{10}O_7$, which was the metabolic product actually isolated, is a colourless substance. The anhydrous substance $C_{10}H_8O_6$, which has not yet been obtained crystalline, has a great affinity for water, being able to abstract it from A.R. chloroform [Clutterbuck, Oxford, Raistrick and Smith, 1932, p. 1454], a fact which suggests that the H_2O is really constitutionally bound to one of the carbonyl groups and that $C_{10}H_{10}O_7$ is in reality only a monoketone (see formula II, second alternative). In support of this view it may be mentioned that $C_{10}H_{10}O_7$ yields only a mono-2:4-dinitrophenylhydrazone (crystallising with $1H_2O$) and even when treated with a large excess of phenylhydrazine yields only the monophenylhydrazone of $C_{10}H_8O_6$, which, however, appears to crystallise from non-hydroxylic solvents without any water of crystallisation. The supposed quinoxaline (p. 643) derived from $C_{10}H_{10}O_7$ is also abnormal, since it crystallises with $1H_2O$ and does not give the expected fluorescence in benzene or alcoholic solution, or any striking coloration with strong sulphuric acid. $C_{10}H_{10}O_7$ (unlike $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_6$) does, however, give a deep yellow solution when dissolved in cold dilute ammonia, so that the ion appears to be coloured although the undissociated acid is colourless. All α-diketones are not coloured substances (*e.g.* *pp'*-diethoxybenzil is colourless, although the compounds $C_6H_5.CO.CO.CH_3$ and



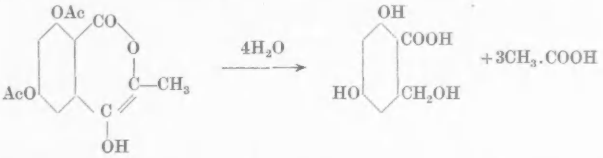
$C_6H_4\begin{cases}COOH\\CO.CO.C_6H_5\end{cases}$

(benzil-*o*-carboxylic acid) are definitely yellow), and the true explanation of the colour of most α-diketones is not yet clear [*cf.* Robinson, 1932, p. 31]. $C_{10}H_8O_6$ appears to be another example of an α-diketone with abnormal properties.

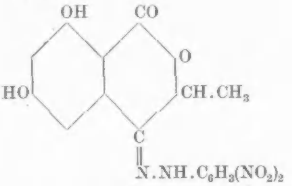
Another fact requiring explanation is the inability, already noted, of $C_{10}H_{10}O_7$ to give a fluorescent solution in cold concentrated sulphuric acid. If the assumption is made that the fluorescence is a property of the *iso*coumarin ring it will be seen that although both $C_{10}H_{10}O_5$ and $C_{10}H_{10}O_6$ are able to lose H_2O to yield *iso*coumarin derivatives, $C_{10}H_8O_6$ cannot yield an *iso*coumarin in this way since in the side-chain —CO.CO.CH_3 there is no hydrogen on the C atom which is directly attached to the benzene ring, and hence $C_{10}H_{10}O_7$ would not be expected to give a fluorescence with concentrated H_2SO_4.

III. *$C_{10}H_{10}O_6$.* The formula suggested for this acid also presents certain apparent difficulties. Repeated recrystallisation of the pure substance from water undoubtedly lowers the point at which melting begins (see experimental part) and although most specimens of $C_{10}H_{10}O_6$ (as Na salt) reduced copper sulphate in the cold, a specimen of the pure metabolic product isolated *via* the Ca salt and recrystallised from acetone-chloroform (not from water) had no effect on copper sulphate in the cold. Further, although $C_{10}H_{10}O_7$ was obtained by the oxidation of a sample of pure $C_{10}H_{10}O_6$ by excess of copper sulphate the yield was very small and most of the $C_{10}H_{10}O_6$ was recovered unchanged. A possible explanation is offered by the fact that there are 5 possible side-chains (see p. 636) of which only —CHOH.CO.CH_3, —CO.CHOH.CH_3 and —C(OH)=C(OH)CH_3 would be likely to reduce cupric salts in the cold, and that $C_{10}H_{10}O_6$ after crystallisation from water is a mixture of tautomerides. This hypothesis may also explain the optical inactivity of the samples of $C_{10}H_{10}O_6$ isolated, since one possible side-chain, *viz.* —C(OH)=C(OH).CH_3, has no asymmetric carbon atom, and this may be the form in which the substance is actually produced by the mould. It may be recalled that the acetylmethylcarbinol isolated from certain bacterial fermentations is also practically optically inactive.

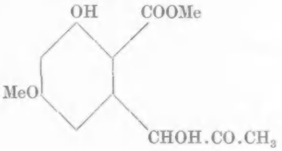
Other properties of $C_{10}H_{10}O_6$ are in good agreement with the suggested structural formula. Acetylation, for instance, yields a neutral acetyl derivative of $C_{10}H_8O_5$ which on hydrolysis yields 3 molecules of acetic acid. It is not quite certain, however, that this derivative is really a triacetyl derivative of $C_{10}H_8O_5$, since the compound obtained when it is hydrolysed by cold alkali appears to have the empirical formula $C_8H_8O_5$. Dieckmann and Meiser [1908] found that *iso*coumarin-4-carboxylic acid was readily split up into formic and homophthalic acids on alkaline hydrolysis so that if the above acetyl derivative is only a diacetyl derivative of $C_{10}H_8O_5$ (the C and H contents of which are practically the same as those of the triacetyl derivative) it might still yield 3 molecules of acetic acid on hydrolysis to give ω-hydroxyorsellinic acid in accordance with the scheme:



Although $C_{10}H_{10}O_6$ (like acetylmethylcarbinol, Clutterbuck, private communication) in aqueous solution does not yield an immediate precipitate with Brady's reagent, the precipitate which slowly forms on long standing analyses correctly for the 2:4-dinitrophenylhydrazone of $C_{10}H_8O_5$ (possibly formula VI). Methylation of $C_{10}H_{10}O_6$ by diazomethane yields a phenolic dimethyl derivative (formula VII).

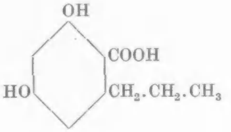


VI



VII

It may be mentioned here that yet another analogy (and the closest so far met with) between mould metabolic products and lichen acids is afforded by comparing the structural unit of the depside divaricatic acid extracted from the lichen *Evernia divaricata* [Hesse, 1911] with the structure proposed for $C_{10}H_{10}O_5$ (I). Divaricatic acid is built up from 2:4-dihydroxy-6-*n*-propylbenzoic acid (VIII) [Hesse, 1911; Mauthner, 1922; 1924; Sonn and Scheffler, 1924; Sonn, 1931; Asahina and Hirakata, 1932] which is theoretically derivable from the mould metabolic product $C_{10}H_{10}O_5$ by the reduction of the $>CO$ group in the side-chain of the latter to $>CH_2$.



VIII

Unfortunately, owing to the ease with which water is eliminated from $C_{10}H_{10}O_5$ to yield $C_{10}H_8O_4$, this reduction appears not to be possible *in vitro* by the ordinary Clemmensen method.

EXPERIMENTAL.

Section I.

(*a*) *Separation of* $C_{10}H_{10}O_5$ *from* $C_{10}H_{10}O_7$ *and relative yields of these products.* It will be recalled (Part XXIV, p. 1452) that these two metabolic products were precipitated together but that no indication was given in that memoir as to their relative yields. Actually the melting-point and behaviour on further heating of the crude fractions containing these substances indicated that, with one exception, *P. stoloniferum* Thom (Ad. 89), $C_{10}H_{10}O_7$ must be the chief constituent. This was confirmed by large-scale fractional crystallisation from water of some 200 g. of mixed $C_{10}H_{10}O_5 + C_{10}H_{10}O_7$ fractions obtained from all the species worked with, with the exception of *P. stoloniferum*, Ad. 89. The yield of $C_{10}H_{10}O_5$ was only 6 g. (3 %), the rest being $C_{10}H_{10}O_7$ together with a considerable amount of gummy material and a very small amount of $C_{10}H_{10}O_6$. In the case of *P. stoloniferum*, Ad. 89, a specimen of the crude fraction had by no means entirely melted at 135° and effervescence was observed at 140–150°; the melt also resolidified after standing for a few days and then had M.P. 120–165°; hence it was concluded that the crude fraction contained much more $C_{10}H_{10}O_5$ than usual. Fractional crystallisation from water, however, revealed that the fraction contained relatively much $C_{10}H_{10}O_6$ and that this was responsible for the anomalous behaviour of the crude fraction on heating. Reference to Table VI in Part XXIV (p. 1451) will indicate that *P. stoloniferum* Ad. 89, gives a larger yield of crude $C_{10}H_{10}O_6$ fractions than any other species. The chief phenolic metabolic products of most species in the *P. brevi-compactum* series are therefore usually mycophenolic acid (if formed at all) and $C_{10}H_{10}O_7$. The yield of $C_{10}H_{10}O_6$ is rather smaller and the yields of $C_{10}H_{10}O_5$ and $C_8H_6O_6$ are very much smaller than that of $C_{10}H_{10}O_7$. With *P. stoloniferum*, Ad. 89, $C_{10}H_{10}O_6$ appears to be the chief metabolic product.

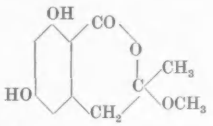
The fractional crystallisation of the mixed $C_{10}H_{10}O_5 + C_{10}H_{10}O_7$ fractions was effected by dissolving in 3 parts of hot water and allowing to stand for several days after seeding with pure $C_{10}H_{10}O_7$. The filtrate from the first crop was allowed to evaporate to about half its volume at room temperature in a vacuum desiccator over calcium chloride. In this way crop 2 and successive crops from successive filtrates were obtained. The first five crops were chiefly $C_{10}H_{10}O_7$, but the last few crops, after seeding with $C_{10}H_{10}O_5$, deposited relatively much $C_{10}H_{10}O_5$. Further purification was effected by dissolving the crude $C_{10}H_{10}O_5$ in a little ethyl acetate and adding boiling 80–100° light petroleum until a marked turbidity was observed and a precipitate began to form. The hot liquid was then filtered through several layers of fluted filter-paper and much boiling 80–100° light petroleum added to the hot filtrate with stirring until precipitation was complete. The almost pure $C_{10}H_{10}O_5$ was filtered off while still hot and a final purification effected by crystallisation from 2 parts of water. The crude $C_{10}H_{10}O_7$ was purified by repeated crystallisation from water (3 parts), the loss being considerable. All the aqueous mother-liquors from the above crystallisations were combined, extracted with ether and the recovered material fractionally crystallised by the same method. This process was repeated as long as the supply of material lasted. No better method of separation was found.

(*b*) *Substance* $C_{11}H_{12}O_5$ *(probably a methyl derivative of* $C_{10}H_{10}O_5$*) isolated during the working up of the metabolic products from* P. brevi-compactum *Dierckx* (P. 75). In Part XXIV (p. 1451) it is mentioned that in the working up of fraction VI the use of alcohol must be avoided since it seemed to lead to partial esterification. In the large scale experiments acetone was employed to dissolve the crude fraction, and from the acetone solution $C_{10}H_{10}O_6$ was precipitated by the addition of much boiling chloroform. In some early experiments, methyl alcohol was used instead of acetone, and it was noticed that the final residue contained an abnormally large content of material insoluble in water. Fractional crystallisation of this insoluble residue from chloroform-light petroleum yielded a new substance in fern-like aggregates of colourless needles, M.P. 173–175° without decomposition.

Micro-analysis (Schoeller): C, 58·95; H, 5·36; OCH_3, 13·42 %. $C_{10}H_9O_4(OCH_3)$ requires C, 58·93; H, 5·40; OCH_3, 13·85 %.

0·0801 g. required 3·60 cc. *N*/10 NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 223. $C_{11}H_{12}O_5$ titrating as a monobasic acid requires an equivalent of 224. The substance is recovered unchanged on acidification of the neutral solution.

The substance is not a true metabolic product, for if acetone is used instead of methyl alcohol, the final residue yields only mycophenolic acid by careful fractional crystallisation from chloroform-light petroleum. $C_{11}H_{12}O_5$ is readily soluble in most organic solvents. It gives an intense but unstable red colour with ferric chloride in alcoholic solution. It is insoluble in water but dissolves immediately in aqueous sodium bicarbonate. It gives no turbidity with Brady's reagent even on standing for an hour, but a copious yellow precipitate forms after standing for a day. The nature of this methyl derivative of $C_{10}H_{10}O_5$ is not quite clear. It appears not to be a ketone, hence is probably not the methyl ester of $C_{10}H_{10}O_5$, but may be a pseudo-ester of the form



[*cf.* Auwers and Heinze, 1919].

(*c*) *Conversion of $C_{10}H_{10}O_5$ into $C_{10}H_8O_4$, and stability of $C_{10}H_8O_4$ towards alkali.* The dehydration of $C_{10}H_{10}O_5$ to give $C_{10}H_8O_4$ is effected:

(i) By heating at 150–170° and crystallisation of the product from water (Part XXIV, p. 1454).

(ii) A more convenient method is to use acetic anhydride as the dehydrating agent and to hydrolyse the resulting diacetyl derivative of $C_{10}H_8O_4$ by cold dilute alkali (see p. 644).

(iii) $C_{10}H_{10}O_5$ is dissolved in water and concentrated H_2SO_4 poured slowly in to make a well-defined bottom layer. A felt of needle-shaped crystals of $C_{10}H_8O_4$ soon forms above the surface of the sulphuric acid.

$C_{10}H_8O_4$ is not readily hydrolysed by *N*/40 NaOH at 70–80°; 0·1146 g. was dissolved in 10 cc. aqueous alcohol and neutralised to phenolphthalein by addition of 5·1 cc. *N*/10 NaOH. (Theoretical for $C_{10}H_8O_4$ titrating as a monobasic acid, 5·95 cc.) A further 5·5 cc. of *N*/10 alkali was added and the solution heated at 70–80° for 40 minutes, when only a further 1·7 cc. was found to have been used up. $C_{10}H_8O_4$ was recovered unchanged by acidifying the solution.

(*d*) *Nature of the compound $C_{10}H_{10}O_6$ after recrystallisation from water.* In Part XXIV, p. 1453, it was recorded that this compound melted at 193–203° with softening, darkening and vigorous gas evolution at the latter temperature. Actually the temperature at which softening begins and also the decomposition point seem to vary from one sample to another, *e.g.* a specimen prepared by the catalytic reduction of $C_{10}H_{10}O_7$ (see later) and crystallised once from water did not soften till 202° (decomp. at 206°) while a specimen of the mould metabolic product after five recrystallisations from water began to soften at 178° (decomp. at 200°). Actually this latter material was quite pure and when again recrystallised yielded a crop of crystals, entirely homogeneous when examined under the microscope and melting as above (178–200°). The mother-liquor from this last crystallisation was also evaporated to dryness at room temperature *in vacuo* over H_2SO_4 and yielded material behaving exactly in the same way on heating. In every case the melt reset on cooling and remelted at 190–200° without effervescence. It may be that the rate of heating and other physical factors affect the temperature at which softening begins, but a possible explanation is that the material, after many recrystallisations from water, is a final equilibrium mixture (see introduction, p. 638).

Section II. Presence of keto-groups. Description of tests and derivatives.

(*a*) *Test for the* —$CH_2.CO.CH_2$— *grouping in* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ *and* $C_{10}H_{10}O_7$. The test for the —$CH_2.CO.CH_2$— grouping as described by Täufel and Thaler [1932] consists in the addition of strong sulphuric acid (2 cc.) to a solution of the ketone (a few mg.) in water (4 cc.) which has been shaken with pure salicylaldehyde to form an emulsion. In spite of purification through the bisulphite compound and fractional distillation, the specimen of salicylaldehyde worked with still gave a distinct red coloration in a blank experiment under these conditions, but the following modification of the test was found to give a perfectly satisfactory result with acetone, and to give an almost negative blank. The ketone (a few mg.) is dissolved in cold 45 % (by weight) H_2SO_4 (5 cc.), purified salicylaldehyde (0·5 cc.) is added and the test-tube placed in warm water and shaken vigorously at half minute intervals, the bath being gradually taken up to 100°. In a very few minutes the upper aldehyde layer develops a deep red, and the lower aqueous layer a yellow colour, while in a blank experiment, even after several hours' heating, the aldehyde layer was only faintly pink, and the aqueous layer was colourless. The results obtained with the three metabolic products were as follows: $C_{10}H_{10}O_5$, strongly positive; $C_{10}H_{10}O_6$, faintly positive, *i.e.* rather pale brownish-red, slowly developing; $C_{10}H_{10}O_7$, negative. $C_{10}H_{10}O_5$ therefore very probably contains the —$CH_2.CO.CH_2$— grouping; $C_{10}H_{10}O_7$ does not contain this grouping, while the doubtful positive result given by $C_{10}H_{10}O_6$ may perhaps be explained by the presence of the grouping —$CHOH.CO.CH_3$.

(*b*) *Mono-2:4-dinitrophenylhydrazone of* $C_{10}H_{10}O_5$. This derivative, which is precipitated immediately, was prepared by adding an excess of Brady's reagent (a solution of 2:4-dinitrophenylhydrazine in 2*N* HCl) to a cold saturated aqueous solution of $C_{10}H_{10}O_5$. The yield was, however, much smaller than with $C_{10}H_{10}O_7$ (*ca.* 20 %) and the compound appears to be appreciably soluble in cold water. It forms orange needles from ethyl acetate-light petroleum, much redder in tint than the corresponding derivative of $C_{10}H_{10}O_7$, and melts at 224–226° with blackening and moderately vigorous effervescence. Micro-analysis (Schoeller). Found: loss at 100° *in vacuo*, nil. C, 49·55, 49·44; H, 3·67, 3·74; N, 13·91, 13·96 %. $C_{16}H_{14}O_8N_4$ requires C, 49·21; H, 3·61; N, 14·36 %.

(*c*) *Behaviour of* $C_{10}H_{10}O_6$ *towards 2:4-dinitrophenylhydrazine; formation of 2:4-dinitrophenylhydrazone of* $C_{10}H_8O_5$ (formula VI). A cold saturated aqueous solution of $C_{10}H_{10}O_6$ gave no immediate precipitate when added to excess of Brady's reagent, but turbidity began to appear after about 30 minutes, and a voluminous yellow precipitate had formed after 3 days. This was collected, dried and crystallised from ethyl acetate-light petroleum; it formed balls of needles, darkening above 200° and melting at 232° with vigorous effervescence and blackening. Found (on air-dried material): C, 49·80, 49·63; H, 3·15, 3·32; N, 13·66 %; loss at 100° *in vacuo*, nil. $C_{16}H_{12}O_8N_4$ (dinitrophenylhydrazone of $C_{10}H_8O_5$) requires C, 49·47; H, 3·12; N, 14·4 %.

Evidently $C_{10}H_{10}O_6$ does not itself form a dinitrophenylhydrazone, but is slowly dehydrated by cold 2*N* HCl to yield a ketone $C_{10}H_8O_5$. This dehydration proceeds rapidly on heating. Thus, if a mixture of an aqueous solution of $C_{10}H_{10}O_6$ and Brady's reagent is heated to boiling and then rapidly cooled, a copious precipitate of the above dinitrophenylhydrazone immediately separates.

(*d*) *Mono-2:4-dinitrophenylhydrazone of* $C_{10}H_8O_6$. To a solution of $C_{10}H_{10}O_7$ (0·23 g.) in water (50 cc.) were added 150 cc. of Brady's reagent. A copious, canary-yellow precipitate formed immediately and was later collected (yield 0·39 g. nearly theoretical), and crystallised from ethyl acetate-benzene or ethyl acetate-light petroleum in yellow rhombs or lemon-yellow leaflets, which darken above 200° and melt sharply at 216° with blackening and very vigorous effervescence. Micro-analysis (Schoeller) on air-dried material: Found, loss at 50° *in vacuo*, 8·0, 8·0 %; $C_{16}H_{12}O_9N_4$, $2H_2O$ requires for the loss of $2H_2O$, 8·2 %. On anhydrous material; Found, C, 47·29, 47·40; H, 2·98, 3·24; N, 13·18 %. $C_{16}H_{12}O_9N_4$ (dinitrophenylhydrazone of $C_{10}H_8O_6$) requires C, 47·52; H, 3·00; N, 13·87 %. This derivative is sparingly soluble in benzene and chloroform, readily soluble in hot ethyl acetate.

(*e*) *Monophenylhydrazone of* $C_{10}H_8O_6$. (i) *From* $C_{10}H_{10}O_7$. A solution of phenylhydrazine hydrochloride (2·0 g., 3 mols.) and hydrated sodium acetate (6·0 g.) in water (20 cc.) was filtered, and to the filtrate was added a solution of pure $C_{10}H_{10}O_7$ (1·3 g., 1 mol.) in water (10 cc.). The mixture was heated on the steam-bath for an hour, when a dark-coloured oil was slowly deposited, which later partially solidified on cooling and was filtered off. The filtrate when acidified with dilute HCl yielded a copious, orange, crystalline precipitate, which was collected, dried and crystallised from ethyl acetate-light petroleum in bunches of yellow needles (0·5 g.); M.P. 214° decomp. after darkening from 200°. Micro-analysis (Schoeller): loss at 100° *in vacuo*, 1·3, 1·6 %; found on material dried at 100° *in vacuo*: C, 61·20; H, 4·64; N, 8·53 %. $C_{16}H_{14}O_5N_2$ requires, C, 61·13; H, 4·49; N, 8·91 %.

The dark-coloured oil precipitated during the course of the reaction solidified on drying *in vacuo* and was then triturated with dilute HCl. The orange-brown powder so obtained (0·67 g.) appeared to consist essentially of the above compound. Although $C_{10}H_{10}O_7$ ($C_{10}H_8O_6$, H_2O) is the hydrated form of a diketone, it appears to be impossible to convert it into an osazone.

(ii) *From* $C_{10}H_{10}O_6$. The conditions were exactly as above, $C_{10}H_{10}O_6$ (1·2 g.) being used instead of $C_{10}H_{10}O_7$. The course of the reaction was also very similar but the dark-coloured oil deposited was later found to consist chiefly of tarry material. The filtrate on acidification yielded a yellow precipitate, which was crystallised from ethyl acetate-light petroleum (0·53 g.); M.P. 213° decomp., alone or mixed with the phenylhydrazone obtained from $C_{10}H_{10}O_7$ (M.P. 214° decomp.). This fact, together with the analytical figures, shows that the two compounds are identical. Found: loss at 100° *in vacuo*, nil; C, 61·10; H, 4·65; N, 8·06 %. $C_{16}H_{14}O_5N_2$ requires C, 61·13; H, 4·49; N, 8·91 %.

(*f*) *The supposed quinoxaline derived from* $C_{10}H_{10}O_7$. A solution of pure $C_{10}H_{10}O_7$ (1 g.) and *o*-phenylenediamine hydrochloride (0·85 g.; slight excess) in alcohol (15 cc.) was boiled under reflux for 30 minutes. The brown liquid was then poured into much 2*N* H_2SO_4 (no precipitate), and dilute caustic potash solution was then cautiously added with stirring until the maximum amount of precipitate appeared to have been formed. The solution was then still strongly acid. The almost colourless amorphous precipitate was collected and dried (1·3 g.) and crystallised from hot water, in which it is very sparingly soluble, in colourless diamond-shaped platelets; M.P. 210° (effervescence and blackening) with marked softening from 200°. Micro-analysis (Schoeller). On air-dried material, C, 60·31, 60·37; H, 4·60, 4·71; N, 8·92, 8·94 %; mol. wt. 270, 267; loss at 100° *in vacuo*, 5·80, 5·84 %. $C_{16}H_{12}O_4N_2$, H_2O requires C, 61·13; H, 4·49; N, 8·92 %; mol. wt., 314; H_2O, 5·73 %. Found on material dried at 100° *in vacuo*: C, 64·05, 64·18; H, 4·20, 4·24; N, 9·06 %. $C_{16}H_{12}O_4N_2$ requires C, 64·84; H, 4·09; N, 9·46 %.

This derivative is very readily soluble in cold ethyl and methyl alcohol, moderately soluble in hot ethyl acetate and almost insoluble in benzene and chloroform. It is readily soluble in sodium bicarbonate solution and gives a pale yellow colour with caustic soda, but is not readily soluble in dilute sulphuric acid. With concentrated sulphuric acid it gives a warm brown coloration, changing to pale yellow on dilution with water. It gives no precipitate with Brady's reagent, and no fluorescence in alcoholic or benzene solution.

Section III. Acetyl derivatives.

(*a*) *Diacetyl derivative of* $C_{10}H_8O_4$. (i) *From* $C_{10}H_{10}O_5$. A solution of $C_{10}H_{10}O_5$ (0·5 g.) and acetic anhydride (5 cc.) in pyridine (6 cc.) was kept at 36° for 6 days, and was then poured into water (250 cc.). The almost colourless precipitate was filtered off (0·5 g.) and crystallised from light petroleum (80–100°) in which it is moderately readily soluble on heating and much more readily soluble in this and other solvents than the triacetyl derivative of $C_{10}H_8O_5$ (*v. infra*). It formed clusters of colourless needles, M.P. 123–124° without decomposition; no coloration was given with ferric chloride in either aqueous or alcoholic solution. Micro-analysis (Schoeller): C, 61·14, 61·05; H, 4·59, 4·38 %. $C_{14}H_{12}O_6$ requires C, 60·85; H, 4·38 %.

This derivative is slowly but completely soluble in cold caustic soda solution, and acidification of the alkaline solution then gives $C_{10}H_8O_4$ in quantitative yield. This property was made use of to prove that the compound contains two acetyl groups. 0·1087 g. was allowed to stand in contact with 28·95 cc. $N/10$ NaOH with frequent shaking for 3 hours when the whole had completely dissolved. 16·68 cc. $N/10$ H_2SO_4 were then required for neutralisation, hence 12·27 cc. $N/10$ NaOH had been neutralised. The end-point to phenolphthalein also corresponded with the commencement of the precipitation of $C_{10}H_8O_4$. If the hydrolysis yields two molecules of acetic acid and one molecule of the monobasic acid $C_{10}H_8O_4$, 11·82 cc. of $N/10$ NaOH should have been neutralised, while if the hydrolysis yields only one molecule of acetic acid, only 9·29 cc. $N/10$ NaOH would be required. By acidifying the titrated solution and filtering off the precipitate, pure $C_{10}H_8O_4$ (0·065 g.; M.P. 247–249°) was recovered (theoretical, 0·076 g.). The derivative therefore is undoubtedly a diacetyl derivative.

(ii) *From* $C_{10}H_8O_4$. $C_{10}H_8O_4$ (0·1 g.) was acetylated by the above method. The product after one recrystallisation from light petroleum (60–80°) had M.P. 119–121°, and M.P. 119–124° when mixed with the diacetyl derivative, M.P. 123–124° prepared from $C_{10}H_{10}O_5$.

(*b*) *Acetylation of* $C_{10}H_{10}O_6$; *formation of a supposed triacetyl derivative of* $C_{10}H_8O_5$. To a solution of pure $C_{10}H_{10}O_6$ (1·0 g.) in pyridine (12·5 cc.) acetic anhydride (10 cc.) was slowly added with shaking and cooling. The flask was corked and kept at 37° for 6 days. The deep brownish-red liquid was then poured into water (250 cc.) and the dark-coloured solid precipitate collected and dried (0·97 g., M.P. 138–154°). After crystallisation from a little benzene, followed by two recrystallisations from light petroleum (80–100°) it formed long, pale yellow, lustrous needles, M.P. 172–174° without decomposition.

Found (micro-analysis by Schoeller): C, 57·67, 57·61; H, 4·28, 4·24; CO.CH_3 (by hydrolysis with H_2SO_4 and distillation of the acetic acid), 38·1 %; CO.CH_3 (by hydrolysis with alcoholic H_2SO_4 and distillation of the ethyl acetate later hydrolysed by standard alcoholic NaOH (A. G. Perkin's method)), 38·4 %. Mol. wt. in camphor, 312, 307, 313; $C_{10}H_5O_2$ (O.CO.$CH_3)_3$ (triacetyl derivative of $C_{10}H_8O_5$) requires C, 57·48; H, 4·22; $COCH_3$, 38·62 %. Mol. wt., 334. The substance gives no coloration with $FeCl_3$ and is neutral but is very slowly soluble in cold $N/10$ NaOH. The product so obtained (pale yellow, feathery needles from chloroform-light petroleum) has M.P. 171–176° (but depressed by admixture with the above triacetyl derivative) and gives a purplish-red coloration with ferric chloride. Micro-analysis (Schoeller): C, 51·10; H, 4·55 %. $C_8H_8O_5$ requires C, 52·15; H, 4·38 %. It is possible that this substance is ω-hydroxyorsellinic acid (see introduction, p. 639).

The triacetyl derivative of $C_{10}H_8O_5$ is readily soluble in chloroform and most other organic solvents, but is only moderately soluble in cold benzene and sparingly soluble in hot light petroleum.

Section IV. Methyl derivatives.

(*a*) *Methylation of $C_{10}H_{10}O_5$ by diazomethane; formation of the monomethyl ether of the methyl ester of $C_{10}H_{10}O_5$.* Pure $C_{10}H_{10}O_5$ (0·8790 g.) was treated with a large excess of ethereal diazomethane in two lots, each prepared from 5 cc. of nitrosomethylurethane. Next day, the ether was evaporated leaving an almost colourless, crystalline product (1·02 g.) which gave an intense purple coloration with ferric chloride in alcoholic solution. It was crystallised from light petroleum (60–80°) in which it is moderately soluble even in the cold, and formed feathery groups of colourless needles, M.P. 88–91°. There was no apparent effervescence due to loss of methyl alcohol on slowly raising the temperature to 220°.

Micro-analysis (Schoeller): C, 60·56, 60·60; H, 5·96, 6·02; OCH_3, 26·22, 26·31 %; $C_{10}H_8O_3(OCH_3)_2$ requires C, 60·48; H, 5·88; OCH_3, 26·05 %.

This dimethyl derivative is insoluble in water and aqueous sodium bicarbonate, but is immediately soluble in dilute caustic potash solution. In aqueous alcoholic solution it gives an immediate copious precipitate with Brady's 2:4-dinitrophenylhydrazine reagent. In alcoholic solution it gives an intense purple coloration with ferric chloride, fading to pale brown in half an hour.

(*b*) *Dimethyl ether of $C_{10}H_8O_4$.* (i) *From the monomethyl ether of the methyl ester of $C_{10}H_{10}O_5$.* A solution of the above dimethyl derivative of $C_{10}H_{10}O_5$ (0·5 g.) and methyl iodide (3 cc.) in acetone (10 cc.) was boiled with finely powdered anhydrous potassium carbonate (3 g.) for 3½ hours. The mixture was then filtered and the filtrate evaporated to dryness *in vacuo*, to yield a partially crystalline brown residue. The crystalline substance was obtained pure by extraction with boiling light petroleum (80–100°) and recrystallisation from the same solvent. It formed clusters of short needles (0·2 g.), M.P. 149–151°.

Micro-analysis (Schoeller): C, 65·24, 65·35; H, 5·41, 5·48; OCH_3, 27·74, 27·55 %; $C_{10}H_6O_2(OCH_3)_2$ requires C, 65·42; H, 5·50; OCH_3, 28·17 %.

This derivative is much more sparingly soluble in light petroleum than the dimethyl derivative of $C_{10}H_{10}O_5$ from which it was prepared. It gives no coloration with ferric chloride and no precipitate with Brady's reagent in alcoholic solution. It is insoluble in cold and slowly soluble in boiling caustic potash, and decolorises a solution of bromine in chloroform instantly.

(ii) *From* $C_{10}H_8O_4$. A solution of $C_{10}H_8O_4$ (1 g.) and methyl iodide (6 cc.) in acetone (30 cc.) was boiled with anhydrous potassium carbonate (4 g.) as above. The product, isolated in exactly the same way, had M.P. 148–150° alone or mixed with the substance obtained from $C_{10}H_{10}O_5$ by the same method, after one recrystallisation from light petroleum. The yield is much superior (0·7 g.) and if a quantity of the substance is required the best method of preparation is to convert $C_{10}H_{10}O_5$ into $C_{10}H_8O_4$ by acetylation and hydrolysis of the diacetyl derivative by cold alkali (p. 644) and to methylate the crude $C_{10}H_8O_4$ directly as above.

(c) *Hydrolysis of the dimethyl ether of* $C_{10}H_8O_4$ *to yield the dimethyl ether of* $C_{10}H_{10}O_5$. 0·0207 g. was boiled under reflux for an hour with 10·00 cc. $N/10$ NaOH. The solution then required 17·9 cc. $N/20$ HCl for neutralisation to phenolphthalein, 2·1 cc. $N/20$ NaOH having been used up (theoretical for the opening of a lactone ring to yield a monobasic acid, 1·9 cc.). The titrated solution was acidified and extracted with ether. Evaporation of the dried ether extract yielded a colourless, crystalline residue, which was recrystallised from light petroleum (80–100°). It formed colourless needles, M.P. 139–141° with slow effervescence at 180°, continuing as the temperature was slowly raised to 220°. Micro-analysis (Schoeller): C, 60·39, 60·52; H, 5·81, 6·06; OCH_3, 26·01, 25·94 %; $C_{10}H_8O_3(OCH_3)_2$ requires C, 60·48; H, 5·88; OCH_3, 26·05 %. 0·1477 g. required 6·22 cc. $N/10$ NaOH for neutralisation to phenolphthalein (sharp end-point) corresponding to an equivalent of 237, the theoretical value for $C_{12}H_{14}O_5$ titrating as a monobasic acid being 238.

This dimethyl ether is but sparingly soluble in high-boiling light petroleum and is partially decomposed by this solvent, the best medium for recrystallisation being chloroform-light petroleum (50–60°). It is sparingly soluble in cold, readily in hot alcohol and in aqueous or alcoholic solution gives no coloration with ferric chloride. It is only moderately soluble in cold ether but readily soluble in cold chloroform. In alcoholic solution it gives an immediate yellow precipitate with Brady's 2:4-dinitrophenylhydrazine reagent. It is immediately soluble in sodium bicarbonate solution.

An attempt to obtain a crystalline oxime failed. The keto-acid (0·0254 g., 1 equiv.) was dissolved in a few cc. of alcohol and hydroxylamine hydrochloride (0·0077 g., 1 equiv.) added together with sufficient $N/10$ NaOH (1·06 cc.) to neutralise all the HCl in the hydroxylamine hydrochloride. The mixture was heated on the water-bath for 30 minutes, allowed to stand for 2 days, then acidified and extracted with ether. Evaporation of the dried ether extract yielded a gum from which nothing crystalline could be obtained.

(d) *Methylation of* $C_{10}H_8O_4$ *by diazomethane; formation of a monomethyl ether* $C_{11}H_{10}O_4$. $C_{10}H_8O_4$ (0·85 g.) was treated with a great excess of ethereal diazomethane (3·6 g.; 20 mols.) in two successive portions, each portion being prepared from 10 cc. nitrosomethylurethane. The reaction was very slow, the evolution of nitrogen being scarcely perceptible, and the material dissolved slowly and with difficulty. The reaction mixture was allowed to stand overnight. Next morning, a test portion still gave a positive ferric chloride reaction. The ethereal solution was shaken with dilute sodium carbonate solution until a test portion no longer gave a coloration with ferric chloride. The alkaline extract was acidified with dilute sulphuric acid and the colourless precipitate collected and dried (0·2 g.) and crystallised from light petroleum B.P. 60–80°) in which it is moderately soluble even in the cold. It formed a coherent felt of colourless feathery needles, M.P. 124–125°.

Micro-analysis (Schoeller): C, 64·12, 64·20; H, 4·92, 4·92; OCH_3, 14·38, 14·93 %. $C_{10}H_7O_3(OCH_3)$ requires C, 64·05; H, 4·89; OCH_3 15·04 %.

This derivative is readily soluble in most organic solvents and is sparingly soluble in boiling water. It is insoluble in sodium bicarbonate solution and is only slowly soluble in sodium carbonate solution. It gives an intense and stable purple coloration with ferric chloride in alcoholic solution.

The ethereal solution after shaking out with sodium carbonate was shaken once with water and dried over anhydrous sodium sulphate. Evaporation of the ether yielded a colourless residue, which was crystallised from light petroleum to yield the pure dimethyl ether of $C_{10}H_8O_4$ (0·6 g.), M.P. 150–152° (p. 645).

(e) *Methylation of $C_{10}H_{10}O_6$ by diazomethane; formation of a dimethyl derivative, probably the monomethyl ether of the methyl ester.* $C_{10}H_{10}O_6$ (0·874 g.) was treated with a large excess of diazomethane and the reaction mixture allowed to stand overnight. The ether was then removed and the product dried to constant weight in a vacuum desiccator. The yield of crude oily product was 1·0235 g., corresponding to the introduction of 2·7 methyl groups. Partial crystallisation occurred after long standing, and the crystalline product was obtained pure by extraction with light petroleum (60–80°) and repeated recrystallisation from low-boiling light petroleum (50–60°). It formed rosettes of colourless, light, feathery needles (19 mg.), M.P. 82°. With alcoholic ferric chloride it gave a pale purplish-brown coloration, fading in a few minutes to brown, finally to orange-brown.

Micro-analysis (Schoeller): C, 56·90; H, 5·49 %; OCH_3, 24·17, 24·14 %. $C_{10}H_8O_4(OCH_3)_2$ requires C, 56·70; H, 5·55; OCH_3, 24·41 %.

This derivative is insoluble in cold water and cold $NaHCO_3$ solution, but is readily soluble in cold caustic soda.

By the working up of mother-liquors, various syrupy, uncrystallisable residues were obtained as by-products during the isolation of the above dimethyl derivative. These all contained about 22–24 % OCH_3, and were probably mixtures of mono-, di- and trimethyl derivatives of $C_{10}H_{10}O_6$.

Methylation of $C_{10}H_{10}O_7$ by the above method yielded a yellow syrup from which nothing crystalline could be isolated, nor could any crystalline derivative be obtained by further methylation of this syrup by the CH_3I-K_2CO_3-acetone method (see p. 645).

Section V. Interconversion of the three acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$.

(a) *Conversion of $C_{10}H_{10}O_7$ into $C_{10}H_{10}O_6$ by catalytic reduction.* Pure $C_{10}H_{10}O_7$ (0·66 g.; entirely freed from $C_{10}H_{10}O_6$ by six recrystallisations from water) was dissolved in a few cc. of water and added to a solution of palladous chloride (0·2 g.) in dilute acetic acid (50 cc.). Norite (1 g., previously strongly heated) was added and a rapid stream of hydrogen passed in during 2 hours, the flask being vigorously shaken by hand from time to time. The mixture was then filtered and the filtrate made strongly acid with dilute sulphuric acid and extracted with ether. The norite residue was also extracted with ether and the combined ether extracts were dried over anhydrous Na_2SO_4. Evaporation of the ether yielded $C_{10}H_{10}O_6$ in almost quantitative yield, M.P. (after crystallisation from water) 202–206° efferv., and when mixed with authentic $C_{10}H_{10}O_6$ (M.P. 202–204° efferv.) the melting-point was 202–204° (efferv.). Found: C, 53·29, 53·09; H, 4·52, 4·51 %. $C_{10}H_{10}O_6$ requires C, 53·07; H, 4·46 %.

The above figures together with the properties of the compound prove beyond all doubt that it is identical with the metabolic product $C_{10}H_{10}O_6$. Thus it gives a transient red colour with ferric chloride in aqueous solution, but a stable reddish-purple colour in alcoholic solution. Its sodium salt reduces copper sulphate quickly in the cold. In dilute solution it gives no turbidity with Brady's reagent except after standing for more than an hour. Its pale green solution in cold concentrated sulphuric acid has a blue fluorescence in ultra-violet light and becomes deep purplish-brown on heating.

(*b*) *Conversion of* $C_{10}H_{10}O_6$ *into* $C_{10}H_{10}O_7$ *by oxidation of the Na salt by copper sulphate in the cold.* $C_{10}H_{10}O_6$ (0·43 g.; many times recrystallised from H_2O) was neutralised by the addition of 30 cc. $N/10$ NaOH. 7 % aqueous copper sulphate solution (24 cc.) was added and the mixture allowed to stand for an hour. Copious precipitation of Cu_2O took place almost immediately, but at the end of an hour, the supernatant liquid was still green in colour and the reaction appeared to have gone to completion. The reaction mixture was filtered, the filtrate acidified with dilute sulphuric acid and extracted with ether. Evaporation of the dried ether extract yielded a pale yellow crystalline solid, most of which was insoluble in boiling chloroform and indeed proved to be unchanged $C_{10}H_{10}O_6$. The hot chloroform extract was diluted with boiling petroleum (60–80°) until a pronounced turbidity was observed and put aside to crystallise, yielding a small amount of pale yellow, flat needles, M.P. 110–132°, with vigorous efferv., raised to 120–133° (efferv.) after one crystallisation from water; yield 0·01 g. Found: C, 48·78; H, 4·39 %. $C_{10}H_{10}O_7$ requires C, 49·57; H, 4·17 %. The substance gave an immediate precipitate with Brady's reagent and was undoubtedly $C_{10}H_{10}O_7$.

Because of the smallness of the yield it was necessary to prove that the original $C_{10}H_{10}O_6$ contained no $C_{10}H_{10}O_7$. 0·5 g. of the same specimen of $C_{10}H_{10}O_6$ was extracted with 200 cc. boiling chloroform, but the minute amount of oily material obtained by evaporation of the chloroform extract gave no immediate precipitate with Brady's reagent. $C_{10}H_{10}O_7$ is therefore a true oxidation product of $C_{10}H_{10}O_6$. The smallness of the yield may be accounted for by the suggestion previously put forward (p. 642) that $C_{10}H_{10}O_6$ after many recrystallisations from water forms a stable equilibrium mixture. If one component only is readily oxidised under the above conditions and the equilibrium is re-established very slowly in the cold, part of the $C_{10}H_{10}O_6$ would be recovered unchanged although the reaction appeared to have proceeded to completion.

(*c*) *Conversion of* $C_{10}H_{10}O_7$ *and* $C_{10}H_{10}O_6$ *into* $C_{10}H_{10}O_5$ *by reduction by zinc dust and dilute sulphuric acid.* Pure $C_{10}H_{10}O_7$ (2·83 g., 6 times recrystallised from H_2O) was dissolved in dilute sulphuric acid (100 cc. of $2N$) by heating, and a few cc. of the solution removed for a control experiment to determine the stability of $C_{10}H_{10}O_7$ towards hot dilute sulphuric acid (actually $C_{10}H_{10}O_7$ was recovered unchanged after boiling the test portion for 3 hours). The bulk of the liquid was maintained at 80–90° while a very large excess of zinc dust was added in small lots with frequent shaking. The whole was then heated on the steam-bath for 2 hours, cooled and filtered. The residual zinc was washed first with dilute sulphuric acid and then several times with water, and the combined filtrates were extracted 5 times with much ether. The dried ethereal extract was evaporated to small bulk and poured into much light petroleum when a pale brown oil separated which later solidified (yield of crude $C_{10}H_{10}O_5$, *ca.* 20 %). After one crystallisation from water (2 parts) it yielded pure $C_{10}H_{10}O_5$ in large colourless tablets, M.P. 150–160° (vigorous efferv.) resets and remelts at 210–222° with slight darkening.

The same product was obtained in rather smaller yield when $C_{10}H_{10}O_6$ was reduced under the same conditions. In this instance, several crystallisations from ethyl acetate-light petroleum followed by one crystallisation from water were needed to effect complete purification (the chief reduction product being an uncrystallisable gum). The substance melted at 152–156° (efferv.) alone or mixed with the reduction product from $C_{10}H_{10}O_7$, or with the authentic metabolic product $C_{10}H_{10}O_5$. In every instance the melt reset and remelted at 210–230°. Found: C, 57·20, 57·06; H, 4·86, 4·90 %. $C_{10}H_{10}O_5$ requires C, 57·14;

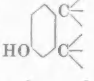
H, 4·76 %. In order to place the matter beyond doubt, the products obtained by heating the two reduction products at 150–170° were each recrystallised from water to give colourless needles, M.P. 242–248°, in each instance, either alone or mixed with an authentic specimen of $C_{10}H_8O_4$, M.P. 244–248°. Micro-analysis (Schoeller) on dehydration product from reduction product of $C_{10}H_{10}O_7$; found: C, 61·89; H, 4·61 %. $C_{10}H_8O_4$ requires C, 62·50; H, 4·20 %.

The zinc dust residues from the reduction of $C_{10}H_{10}O_7$ yielded, by dissolution in dilute sulphuric acid and extraction with ether, a small amount of $C_{10}H_8O_4$.

(*d*) *Reduction of* $C_{10}H_{10}O_7$ *and* $C_{10}H_{10}O_5$ *by Clemmensen's method: formation of* $C_{10}H_8O_4$. $C_{10}H_{10}O_7$ (3 g.; freed from $C_{10}H_{10}O_5$ by 5 recrystallisations from water) was dissolved in 350 cc. 12 % hydrochloric acid and added to amalgamated zinc (300 g.). The whole was boiled under reflux for 3½ hours. During the course of the reduction a considerable amount of colourless material sublimed and condensed on the lower surface of the condenser cork. This was scraped off and crystallised from water and proved to be $C_{10}H_8O_4$ (M.P. 230–247° alone or mixed with authentic $C_{10}H_8O_4$). The aqueous reaction liquid was decanted from the zinc and extracted with ether. Evaporation of the ether yielded a brown semi-solid product which was treated with boiling water (500 cc.). The aqueous extract deposited, on cooling, impure $C_{10}H_8O_4$ mixed with tarry material. The sandy residue unextracted by water was crystallised from much water yielding well formed hexagonal prisms, M.P. 234–248°, alone or mixed with authentic $C_{10}H_8O_4$. The total yield of $C_{10}H_8O_4$ was small, but no other crystalline product could be isolated.

The reduction of $C_{10}H_{10}O_5$ (2 g. crystallised from H_2O to remove $C_{10}H_{10}O_7$) was carried out under similar conditions but the method of working up the crude product was somewhat modified. The hot supernatant liquid was decanted and the zinc was washed with a little water, the washings being added to the supernatant liquid. To this was also added the deposit on the cork, and the whole was placed in the ice-chest overnight. The copious, colourless crystalline precipitate was collected (0·18 g.) and crystallised from water; M.P. 240–248° (darkening) alone or mixed with authentic $C_{10}H_8O_4$. When mixed with the product, M.P. 234–248°, obtained by the Clemmensen reduction of $C_{10}H_{10}O_7$, the M.P. was 236–248°. Found: C, 62·33, 62·39; H, 4·35, 4·30 %. $C_{10}H_8O_4$ requires C, 62·50; H, 4·20 %. The aqueous filtrate from the above and also the residual zinc were extracted with ether and yielded 1·18 g. of a partly crystalline paste from which $C_{10}H_8O_4$, but no other crystalline material, was isolated.

Section VI. Proof that the three acids contain the structure



(*a*) *Oxidation of* $C_{10}H_{10}O_7$ *by* H_2O_2 *in alkaline solution; formation of 3:5-dihydroxyphthalic acid.* Pure $C_{10}H_{10}O_7$ (0·3 g.) was dissolved in a few cc. of sodium bicarbonate solution and a very large excess of hydrogen peroxide solution added. The deep brown solution was heated on the water-bath for about an hour, acidified and extracted once with ether. Evaporation of the dried ether extract yielded a dark oil, which was dissolved in a little ethyl acetate and much benzene added. After standing for several days, a little brown crystalline material had been deposited, which after crystallisation from ethyl acetate-light petroleum yielded a minute amount of almost colourless crystalline material (plates), M.P. 192° (efferv.), reset and remelted at 200–206°. Mixed with authentic 3:5-dihydroxyphthalic acid the M.P. was 190–192° (efferv.) reset and remelted at

200–208°. The oxidation product, which gave a deep slightly purplish-red coloration with ferric chloride in aqueous solution is therefore undoubtedly 3:5-dihydroxyphthalic acid.

(*b*) *Oxidation of the dimethyl ether of* $C_{10}H_8O_4$ *by cold alkaline* $KMnO_4$*; formation of 3:5-dimethoxyphthalic acid.* The pure dimethyl ether of $C_{10}H_8O_4$ (0·05 g.) was suspended in 10 cc. *N* NaOH and 5 % aqueous potassium permanganate solution added, at first drop by drop with shaking (the purple colour being quickly destroyed), later in large excess with occasional shaking. After filtration from MnO_2, decoloration of the filtrate by SO_2, extraction with ether and evaporation of the dried ether extract, a colourless solid product was obtained which was dissolved in a little hot water and the aqueous solution allowed to stand in the ice-chest overnight. Small colourless prisms separated (0·02 g.), which were dried in air, and their behaviour on heating was carefully observed. Two crystals placed in a melting-point tube became opaque between 100 and 130°, but nothing further happened till 180–182° was reached, when effervescence took place. The temperature was taken to 194°, and the tube then removed from the bath when the melt immediately reset and then remelted at 142–148°. The recorded decomposition-point of 3:5-dimethoxyphthalic acid to give the anhydride is 158° [Oxford and Raistrick, 1932], the second melting-point, *i.e.*, of the anhydride, being 143–148°. In order to clear up this discrepancy the behaviour of a single crystal of authentic 3:5-dimethoxyphthalic acid on heating was noted, when it was observed that no effervescence took place till 184° was reached, although when a melting-point was taken in the ordinary way using a column of substance about 2 mm. deep in the melting-point tube, effervescence did occur at 158°. The melting-point of a mixture of the oxidation product with authentic 3:5-dimethoxyphthalic acid, the temperature being slowly raised, was 160–162° (efferv.) the second melting-point being 144–149°. A second mixture had M.P. about 160° (efferv.), the second melting-point being 142–148°. There can thus be no doubt that the oxidation product is in fact 3:5-dimethoxyphthalic acid, a conclusion confirmed by a complete micro-analysis (Schoeller). Found: loss at 100° *in vacuo* 8·2, 8·1 %; $C_8H_4O_4(OCH_3)_2, H_2O$ requires H_2O, 7·4 %. Found (on anhydrous material): C, 53·36, 53·31; H, 4·66, 4·54; OCH_3, 26·77 %. $C_8H_4O_4(OCH_3)_2$ requires C, 53·08; H, 4·45; OCH_3, 27·41 %.

The same substance was obtained in smaller yield when the oxidation by alkaline permanganate was carried out at 100°.

(*c*) *Action of heat on the dimethyl ether of* $C_{10}H_{10}O_5$*. Formation of a neutral ketone yielding 3:5-dimethoxybenzoic acid on oxidation by alkaline permanganate.* The dimethyl ether of $C_{10}H_{10}O_5$ ($C_{12}H_{14}O_5$) (0·3310 g.) was heated in a slow stream of dry and CO_2-free N_2 at 180–210° until effervescence had ceased. The gaseous reaction products were passed first through a weighed $CaCl_2$ U-tube and then through two bubblers each containing 20 cc. *N*/5 baryta. The water given off weighed 0·0250 g. (0·9 equivalent) and the CO_2 given off was equivalent to 3·3 cc. *N*/10 baryta (*i.e.* 0·0073 g.; 0·1 equivalent). The melt, which was dark in colour and reset on cooling, weighed 0·2977 g. so that the loss in weight was 0·0333 g. while the combined weights of H_2O and CO_2 absorbed totalled 0·0323 g. The melt was essentially the dimethyl ether of $C_{10}H_8O_4$ and on hydrolysis by means of boiling *N*/10 NaOH yielded $C_{12}H_{14}O_5$ identical in all respects with the starting material. It was noticed, however, that a small amount of colourless oil had sublimed and condensed on the cool part of the tube in which the heating had been carried out. The top part of the tube containing this oil was therefore cut off and the oil dissolved in water. To a small portion of the solution was added an excess of 2:4-dinitrophenylhydrazine reagent when an immediate turbidity, later changing to a yellow precipitate was produced. The rest of the solution was made alkaline and several cc. of 5 % $KMnO_4$ solution were added. After standing for several hours, the liquid was decolorised by SO_2 and extracted with ether. Evaporation of the dried ether extract yielded a minute amount of a pale yellow solid which was crystallised from water to yield a few ill-defined crystals, M.P. 174–178°, with partial sublimation in the form of colourless needle-shaped crystals which melted sharply at 178–180°.

The experiment was repeated starting from 0·8 g. of $C_{12}H_{14}O_5$. The sublimate on the cool part of the tube was dissolved in water as before, and the melt was hydrolysed by boiling with excess of $N/10$ NaOH for an hour. The hydrolysis liquid was then made strongly alkaline and extracted twice with ether, the residual aqueous solution being then acidified and extracted with ether for the recovery of $C_{12}H_{14}O_5$. The recovered substance, after crystallisation from chloroform-light petroleum, was heated and the products worked up as before. After 4 such heatings, the weight of recovered $C_{12}H_{14}O_5$ was only 0·13 g. Oxidation of the combined aqueous solutions of the oily sublimate by alkaline permanganate yielded only a trace of material which sublimed at about 200° to yield needles of M.P. 176–178°. The combined ether extracts of the alkaline hydrolysis solutions were evaporated to yield a small amount of a yellow oil, which was dissolved in much water and filtered from a little insoluble material. A portion of this solution when treated with Brady's reagent yielded a 2:4-dinitrophenylhydrazone, M.P. 131–132° after crystallisation from ethyl acetate-light petroleum, which was insoluble in dilute alkali and hence could not have been the 2:4-dinitrophenylhydrazone of the starting material $C_{12}H_{14}O_5$. The remainder of the aqueous solution of the neutral ketone was oxidised by alkaline permanganate in the cold, and yielded about 1 mg. of a substance (after crystallisation from water) which was readily soluble in sodium bicarbonate solution, and which melted with partial sublimation at 174–176°, the sublimate (colourless needles) melting at 178–179°. A mixture of this product with an authentic specimen of 3:5-dimethoxybenzoic acid (M.P. 181–182°; sublimate melting at 181°) melted at 176–182°, the sublimate melting at 180°.

It is thus obvious that in the molecule of $C_{10}H_{10}O_5$, and hence also in $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$, the carboxyl group is attached directly to the benzene ring. Also, since the oxidation of the neutral ketone obtained by the decarboxylation of the dimethyl ether of $C_{10}H_{10}O_5$ yields 3:5-dimethoxybenzoic acid the 3-carbon ketonic side-chain must be *meta* to the phenolic OH groups in these acids. In the light of evidence previously produced this side-chain must be in the *ortho* position to the carboxyl group attached directly to the benzene ring.

(*d*) *Behaviour of the dimethyl ether of* $C_{10}H_{10}O_5$ *towards alkaline iodine* [cf. *Birkinshaw and Raistrick, 1931*]. *Proof of the presence of the side-chain* $—CH_2.CO.CH_3$. The pure ether (11·3 mg.) was dissolved in a few cc. of $N/10$ NaOH, and water (70 cc.) added, followed by $N/10$ iodine (40·0 cc. accurately measured from a pipette) and $N/10$ NaOH (50 cc.). The flask was corked and allowed to stand. A turbidity was noticed within 5 minutes and precipitation of iodoform appeared to be complete within 30 minutes. At the end of 2 hours, $2N$ H_2SO_4 (10 cc.) was added and the unused iodine titrated against $N/10$ thiosulphate when it was found that 4·47 cc. $N/10$ iodine had been used up, corresponding to 9·4 atoms of iodine per molecule of $C_{12}H_{14}O_5$. The yellow crystalline precipitate was filtered off and was undoubtedly iodoform, having M.P. 116–120°.

Similar experiments were carried out with propiophenone (redistilled B.P. 215–216°) and with benzylmethylketone (redistilled B.P. 214–216°). The first named ketone (9·8 mg.) oxidised under conditions exactly similar to those outlined above, used up only 1·48 cc. *N*/10 iodine (*i.e.* 2·0 atoms per molecule of propiophenone) and no trace of iodoform or any other insoluble material was formed. The parallel experiment with benzylmethylketone was carried out under the following conditions. The ketone (8·4 mg.) was dissolved in water (20 cc.) and *N*/10 iodine (60·0 cc.) was added, followed by *N*/10 NaOH (75 cc.). The mixture was allowed to stand for 2½ hours, but separation of iodoform appeared to be complete within the first 30 minutes. 5·49 cc. *N*/10 iodine were used up, corresponding to 8·8 atoms of iodine per molecule of ketone. Since the formation of iodoform and the substituted acetic acid from a substituted acetone requires only 6 atoms of iodine, it is evident that both with $C_{12}H_{14}O_5$ and with benzylmethylketone the oxidation proceeds beyond this stage, probably to the corresponding benzoic acid if a sufficiently large excess of iodine is allowed to act on the ketone for a long enough time. In a control experiment with 3:5-dimethoxyphthalic acid hydrate (14·2 mg.) under the conditions described above for the oxidation of $C_{12}H_{14}O_5$, only 0·08 cc. *N*/10 iodine had been used up in the course of 2 hours, and no iodoform was produced.

It is thus very probable that the 3-carbon side-chain in $C_{10}H_{10}O_5$ is $—CH_2.CO.CH_3$ as in benzylmethylketone and not $—CO.CH_2.CH_3$ as in propiophenone.

(*e*) *Conversion of the dimethyl ether of* $C_{10}H_8O_4$ ($C_{12}H_{12}O_4$) *into* $C_{12}H_{13}O_3N$, *presumably 6:8-dimethoxy-3-methyl*iso*carbostyril* (*Formula V*) [cf. *Bamberger, 1892*]. A mixture of $C_{12}H_{12}O_4$ (0·4 g.) and saturated ethyl alcoholic ammonia (13 cc.) was heated in a sealed tube for 4 hours at 120–160°. The alcohol was then removed by evaporation, finally *in vacuo*, leaving a brown, solid product which was dissolved in a little cold chloroform, and boiling light petroleum (60–80°) was added until a pronounced flocculent precipitate had formed. The hot solution was then filtered and more hot light petroleum added to the filtrate. Fan-shaped clusters of needles soon began to separate, which when collected and dried in air became slightly brown in colour; M.P. 216–218° without decomposition.

Micro-analysis (Schoeller): C, 65·24, 65·46; H, 6·01, 6·02; N, 6·43, 6·51; OCH_3, 27·51 %. $C_{10}H_7ON(OCH_3)_2$ requires C, 65·72; H, 5·98; N, 6·39; OCH_3, 28·30 %.

This substance is readily soluble in cold chloroform, moderately soluble in hot water, only sparingly soluble in cold ethyl alcohol or acetone, and practically insoluble in hot light petroleum and cold water. The aqueous solution gives no coloration with ferric chloride. The substance is a feeble base, dissolving slowly in cold, dilute hydrochloric acid, but is quite insoluble in cold caustic soda solution. It gives no coloration with cold concentrated sulphuric acid, but a deep red colour develops on adding a trace of potassium dichromate.

SUMMARY.

The three acid metabolic products, containing ten carbon atoms, of *P. brevi-compactum* Dierckx and related species, *viz.* $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, and $C_{10}H_{10}O_7$ have been shown to be 3:5-dihydroxy-2-carboxybenzylmethylketone, 3:5-dihydroxy-2-carboxyphenylacetylcarbinol (or a mixture of its tautomerides) and a hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone respectively. They are therefore closely related constitutionally to each other, to 3:5-dihydroxyphthalic acid and to mycophenolic acid (other metabolic products of the same series of fungi), and to the lichen acid divaricatic acid.

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LXXXVI. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XXXI. THE MOLECULAR CONSTITUTION OF THE METABOLIC PRODUCTS OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX AND RELATED SPECIES.

II. MYCOPHENOLIC ACID.

By PERCIVAL WALTER CLUTTERBUCK and
HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

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Whilst engaged on a study of the deterioration of Italian maize by mould infections, in the belief that pellagra is caused by the toxic substances present in mouldy maize, Gosio [1896] isolated a strain of *Penicillium* which he named *P. glaucum* but which was almost certainly one of the *P. brevi-compactum* series. When this species was grown on maize itself or on Raulin's medium a metabolic substance was obtained which melted at 145° and gave an intense blue colour with ferric chloride. Gosio assigned to it the formula $C_9H_{10}O_3$, based on a single combustion and no mol. wt. determination. Alsberg and Black [1913] isolated from mouldy Italian maize a strain of *Penicillium stoloniferum* Thom (*P. stoloniferum* may be regarded as a synonym of *P. brevi-compactum* Dierckx), which when grown on Raulin's medium gave the characteristic ferric chloride reaction of Gosio. They isolated the metabolic product responsible for this reaction and called it mycophenolic acid. This acid was obtained in the form of colourless needles melting at 140°, and the formula $C_{17}H_{20}O_6$ was assigned, deduced from analysis, titration value and molecular weight estimations. It seems probable that the acid isolated by Gosio is identical with Alsberg and Black's mycophenolic acid. Recently Clutterbuck, Oxford, Raistrick and Smith [1932], in a study of the metabolic products of fifteen species and strains in the *Penicillium brevi-compactum* Dierckx series, isolated mycophenolic acid, together with the following new mould metabolic products; $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$, and an acid $C_8H_6O_6$ which has been shown by Oxford and Raistrick [1932] to be 3:5-dihydroxyphthalic acid. Mycophenolic acid was obtained from the metabolism solutions of twelve of these species and strains and it was found that the organisms tended to lose their power to form mycophenolic acid during artificial cultivation over long periods (20 years).

During this work considerable amounts of mycophenolic acid were accumulated, and it is the purpose of this paper to describe the investigation of the molecular constitution of this acid. The molecular constitutions of the acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ are dealt with in the previous paper [Oxford and Raistrick, 1933].

Properties of mycophenolic acid.

Mycophenolic acid is almost insoluble in cold water but crystallises beautifully in radiating colourless needles from boiling water or from aqueous alcohol and melts at 141°. It is very soluble in alcohol, moderately so in ether and chloroform, less soluble in benzene and cold toluene. The lead, silver and copper salts are amorphous and insoluble in water. The disodium and dipotassium salts are very soluble in water but the dipotassium salt is insoluble in absolute alcohol. The acid forms two series of salts, *e.g.* with baryta a moderately soluble barium salt $(C_{17}H_{19}O_6)_2Ba$ and an insoluble salt $C_{17}H_{18}O_6Ba$. With ferric chloride it gives a very intense and stable pure blue colour in alcoholic solution and a blue-violet colour in aqueous solution. It gives a heavy precipitate with bromine water but no reaction with bleaching powder solution. It is very stable to boiling acids and to boiling alkalis, being unchanged by heating in nitrogen in 45 % KOH for 6 hours at 140°. It does not reduce Fehling's solution or ammoniacal silver nitrate but is immediately oxidised by permanganate in the cold and is readily reduced catalytically by palladium and hydrogen at ordinary temperatures and pressures. Mycophenolic acid dissolved in cold concentrated H_2SO_4 does not give a blue fluorescence in ultra-violet light, as do the metabolic products $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_5$. It can be extracted slowly from aqueous solution at p_H 7 with ether, but titrates as a dibasic acid. It is optically inactive (1·0106 g. in 40 cc. absolute alcohol in a 4 dm. tube: mean of 20 readings + 0·02°) and contains a methoxyl group.

The following results were obtained by micro-combustion (Schoeller): C, 63·85, 63·80; H, 6·36, 6·33; OCH_3, 9·65, 9·36 %; mol. wt., 337, 341. $C_{17}H_{20}O_6$ requires C, 63·73; H, 6·30; OCH_3, 9·69 % and mol. wt., 320·2.

Also 0·0930 g. mycophenolic acid required 5·49 cc. *N*/10 NaOH for neutralisation in the cold to phenolphthalein, corresponding to an equivalent of 169 (theoretical for $C_{17}H_{20}O_6$ titrating as a dibasic acid = 160).

Presence of a methoxyl group in mycophenolic acid.

The micro-Zeisel determination above showed that one alkoxyl group was present. The characterisation of this as a methoxyl group was carried out by passing the products from a macro-Zeisel determination into freshly distilled dimethylaniline. Crystals (plates) separated which were left to stand overnight, filtered off and dried over H_2SO_4 *in vacuo*. A synthetic sample of trimethylphenylammonium iodide was also prepared. These two samples and a mixed sample, on heating in sealed tubes, all sintered at 213° and completely volatilised at 220°. An iodine estimation was then carried out on the product from mycophenolic acid by precipitating the iodine with silver nitrate in aqueous solution in presence of nitric acid. The silver iodide was collected on a Gooch filter, dried and weighed. 0·1285 g. of the trialkylphenylammonium iodide from mycophenolic acid gave 0·1150 g. AgI corresponding to 48·38 % of iodine. Theoretical for the methyl compound = 48·3 %.

Mycophenolic acid therefore contains one methoxyl group.

nor*Mycophenolic Acid* ($C_{16}H_{18}O_6$) *by demethylation of mycophenolic acid with HI.*

Mycophenolic acid (1 g.) and HI (20 cc.) were heated in a stream of CO_2. The temperature was gradually raised and the volatile products passed through excess alcoholic $AgNO_3$ as in the usual Zeisel technique. The first appearance of a precipitate in the silver nitrate bubbler occurred with a bath temperature of 80°.

The mixture was therefore maintained at 85° for 3 hours, the reaction being then practically complete as indicated by the weight of AgI. The hydriodic acid solution was then poured into water and allowed to stand overnight. The separated acid was filtered off, dissolved in ether and shaken with dilute thiosulphate solution to remove iodine, the ethereal extract evaporated and the solid acid washed with water and dried. 0·92 g. of an acid beginning to soften at 175° and melting at 181° was obtained. The acid was sublimed and gave a product, M.P. 184°. It was finally recrystallised from benzene-light petroleum and separated in needles, M.P. 184°, which gave the following results on micro-combustion (Schoeller): C, 62·85, 63·02; H, 6·06, 6·05 %; OCH_3, nil; mol. wt., 303, 306. $C_{16}H_{18}O_6$ requires C, 62·73; H, 5·93 %; mol. wt., 306. Also, 0·0249 g. required 1·60 cc. $N/10$ NaOH corresponding to an equivalent of 155·6 (theoretical for $C_{16}H_{18}O_6$ titrating as a dibasic acid = 153·1).

*nor*Mycophenolic acid gives in alcoholic solution a greyish-blue colour with a trace of $FeCl_3$, a green colour with excess, and in aqueous solution a blue-violet colour.

Acetylation of mycophenolic acid.

0·3 g. of mycophenolic acid was heated under reflux for ½ hour with 0·6 g. sodium acetate and 1·2 cc. acetic anhydride, cooled and diluted with water, acidified with dilute H_2SO_4 and extracted with ether. On evaporating the ether solution an oil separated and was dissolved in glacial acetic acid; water was added to turbidity and the solution allowed to stand 3 days. The material gradually crystallised, was filtered off, washed with water, dried *in vacuo* over NaOH and weighed (0·265 g.). After recrystallisation from water it softened at 155° and melted at 158–160°. Acetylmycophenolic acid is soluble in cold aqueous sodium bicarbonate solution.

The following figures were obtained by micro-combustion (Schoeller): C, 63·01, 62·75; H, 6·12, 6·23; OCH_3, 8·70, 8·65 %; mol. wt., 375, 382. $C_{16}H_{16}O_4(OCH_3)(OCOCH_3)$ requires C, 62·94; H, 6·12; OCH_3, 8·57 %; mol. wt., 362·2.

0·1011 g. on titration in cold solution required 2·80 cc. $N/10$ NaOH corresponding to an equivalent of 361 (theoretical for $C_{16}H_{16}O_4(OCH_3)(OCOCH_3)$ titrating as a monobasic acid, 362), and on heating for 1 hour on the water-bath with excess $N/10$ NaOH required 8·30 cc. $N/10$ NaOH. Complete hydrolysis had therefore occurred and the products of hydrolysis required three equivalents of alkali for neutralisation.

Mycophenolic acid contains therefore one acetylatable OH group and since the acetyl compound gives no colour with ferric chloride this OH group is probably phenolic in nature, and is responsible for the characteristic blue colour with ferric chloride.

Hydrolysis of mycophenolic acid with N *NaOH.*

1·015 g. mycophenolic acid (pure, dried over P_2O_5, M.P. 141°) were weighed into a hydrolysis flask fitted at a ground glass joint with a reflux condenser, and titrated to phenolphthalein with N NaOH. When slightly more than half the alkali required for titration had been added, the substance passed completely into solution. A total of 5·73 cc. N NaOH was used. An excess of N NaOH making the total volume used equal to 50 cc. was added and the solution boiled for 7 hours, the condenser being fitted with a soda-lime guard tube and the oil-bath being maintained at 130–135°. The solution was then cooled and the excess of *N* NaOH determined. 43·80 cc. of *N* acid were required. Thus the amount of alkali required for the titration had increased from 5·75 cc. to 6·20 cc. during heating. (Theory for $C_{17}H_{20}O_6$, titrating as a dibasic acid = 6·08 cc.)

In the first experiment the solution was then acidified, the separated oil allowed to crystallise, filtered off and dried. It melted at 137°, gave a blue ferric chloride reaction, and after recrystallisation from boiling water melted at 141°, and was in fact unchanged mycophenolic acid. In the later experiments, however, the neutralised solution after hydrolysis was shaken with ether and rather less than the theoretical amount of *N*/10 acid gradually added with shaking. The ethereal extract was then separated and filtered through a dry filter-paper, the volume somewhat reduced in a stream of air and then poured into 2 vols. of light petroleum. On driving a current of air over the solution, hydrolysed mycophenolic acid separated in needles, which gave the same colours with $FeCl_3$ in alcoholic and aqueous solution as did mycophenolic acid.

Action of heat on hydrolysed mycophenolic acid.

After air-drying for 5 days, 0·6911 g. of hydrolysed mycophenolic acid was heated in an oil-bath in a stream of dry nitrogen, the vapour formed being passed through weighed $CaCl_2$ tubes (filled with nitrogen) and then through baryta. At a temperature of 90°, the volume of the material suddenly decreased considerably and water obviously separated. The bath temperature was not raised above 120° and the substance did not melt. The material lost 0·0397 g. in weight, the $CaCl_2$ tube gained 0·0391 g., and no CO_2 was formed. The loss in weight is thus 5·66 % (theory for $C_{17}H_{22}O_7 \rightarrow C_{17}H_{20}O_6$, 5·33 %). The heated material gave a blue ferric chloride reaction, melted at 139°, and, after recrystallisation from water, at 141°, and did not depress the M.P. of pure mycophenolic acid.

The small increase in titration on warming neutralised mycophenolic acid with excess NaOH and the loss of water by hydrolysed mycophenolic acid with reversion to mycophenolic acid suggest that the acid contains a lactone ring which opens up for the most part, but not completely, during ordinary titration in the cold. Strong support for this conclusion is obtained when the methyl ether of mycophenolic acid is hydrolysed in the same way.

Methylation of mycophenolic acid with diazomethane. The methyl ester of the methyl ether of mycophenolic acid.

Mycophenolic acid (1 g.) was dissolved in dry ether and an excess of ethereal diazomethane added. An immediate reaction took place. After standing overnight, the excess of diazomethane and the ether were removed. The product separated in needles, M.P. 57°. It was recrystallised from aqueous alcohol and melted at 58°.

The following figures were obtained by micro-combustion (Schoeller): C, 65·60, 65·46; H, 6·95, 7·02; OCH_3, 26·44, 26·30 %; mol. wt., 346, 344. $C_{15}H_{15}O_2(OCH_3)_2COOCH_3$ requires C, 65·46; H, 6·95; OCH_3, 26·73 %; mol. wt., 348.

The product is insoluble in NaOH and does not give a ferric chloride reaction. Since it is readily hydrolysed by boiling with alkalis, losing one OCH_3 group and giving the methyl ether of mycophenolic acid, the above product is, therefore, the methyl ester of the methyl ether of mycophenolic acid.

Preparation of the methyl ether of mycophenolic acid by hydrolysis of the above ester.

1·2342 g. of the above ester were heated with 50 cc. *N* NaOH in an oil-bath at 130° for 7 hours. The ester dissolved very slowly. The cooled solution required 43·05 cc. *N* H_2SO_4 for neutralisation to phenolphthalein. During hydrolysis 6·95 cc. *N* NaOH were therefore used (the theoretical amount, assuming that the methyl ether of mycophenolic acid is dibasic, is 6·82 cc.). The neutralised solution was filtered and acidified. The methyl ether separated as an oil which rapidly crystallised, wt. 1·10 g., M.P. 107–108°, which on recrystallisation from boiling water separated in needles, M.P. 112°. It did not give a colour with ferric chloride. The following figures were obtained by micro-combustion (Schoeller): C, 64·84, 64·74; H, 6·83, 6·78; OCH_3, 18·29, 18·31 %; mol. wt., 370, 377. $C_{16}H_{16}O_4(OCH_3)_2$ requires C, 64·65; H, 6·64; OCH_3, 18·57 % and mol. wt., 334·2.

0·1585 g. of the acid required 4·77 cc. *N*/10 NaOH for neutralisation to phenolphthalein in the cold corresponding to an equivalent of 332·4 (theory for $C_{16}H_{16}O_4(OCH_3)_2$ titrating as a monobasic acid = 334). The methylated acid titrates in the cold sharply therefore as a monobasic acid. When the neutralised solution was heated with excess (20 cc.) of *N*/10 NaOH in the water-bath for 1 hour, cooled and titrated back, a further quantity of 4·85 cc. of *N*/10 NaOH was used, the acid thus becoming dibasic.

This behaviour, together with the behaviour of mycophenolic acid itself, confirms the view that mycophenolic acid contains a lactone ring in addition to a carboxyl group. The lactone ring opens almost completely during titration of mycophenolic acid in the cold, but is sufficiently stabilised in the methyl ether to require warming or long standing with NaOH in order to open it.

Acidification of the solution of the hydrolysed methyl ether of mycophenolic acid even when carried out as for the isolation of hydrolysed mycophenolic acid, always resulted in the closure of the lactone ring. Although it was therefore impossible to isolate the hydrolysed methyl ether of mycophenolic acid, its disilver salt was readily obtained by adding silver nitrate to the neutralised hydrolysed solution. The precipitated silver salt, after washing with water and then with alcohol, contained after air-drying 38·52 % Ag. (Theory for $C_{16}H_{22}O_3(COOAg)_2$ = 38·17 %.)

Attempted methylation of hydrolysed mycophenolic acid.

An air-dried sample of hydrolysed mycophenolic acid was treated with diazomethane in the hope of esterifying the second carboxyl group, and possibly of methylating the hydroxyl group, if phenolic, of the opened lactone ring. The same methyl ester of the methyl ether of mycophenolic acid was, however, obtained as in the methylation of mycophenolic acid itself, and it appears, therefore, that treatment with diazomethane closes the lactone ring.

Methylation of mycophenolic acid with methyl sulphate in alkaline solution.

Methylation of mycophenolic acid in alkaline solution with methyl sulphate in the usual way was next tried in the hope that, since the lactone ring would remain open, the lactonic hydroxyl if phenolic would be methylated. Here again, however, the product was the methyl ether of mycophenolic acid, identical with the acid obtained in the experiments with diazomethane. Thus it melted at 112°, did not give a colour with ferric chloride and did not depress the M.P. of the sample previously prepared. It seems probable, therefore, that the lactonic hydroxyl group is not phenolic.

Oxidation of the methyl ether of mycophenolic acid with alkaline $KMnO_4$.

The method followed was essentially that of Luff, Perkin and Robinson [1910]. 4·25 g. of the methyl ether of mycophenolic acid were dissolved in a little KOH, the volume made up to 75 cc. with water and heated to boiling under reflux. Permanganate (260 cc. of a solution containing 5 g. $KMnO_4$ and 2·5 g. KOH per 100 cc.) was added slowly until the disappearance of the pink colour took more than 3 minutes. The solution was then cooled and filtered. Neither the alkaline filtrate nor the MnO_2 sludge after dissolution in sulphurous acid contained any ether-extractable material. The alkaline filtrate was then neutralised, reduced to 200 cc. *in vacuo* at 45°, cooled and made acid to Congo red by addition of 19 cc. concentrated HCl. Nothing separated on standing overnight, and the solution was therefore subjected to continuous ether extraction for 3 days. The material extracted after removing the ether and drying overnight *in vacuo* over H_2SO_4 weighed 3·04 g. It smelled strongly of acetic acid and was therefore dried over KOH, the final weight being 2·9 g. The product was a colourless, very viscous mass, which on stirring with a small volume of dry ether gave 1·19 g. of a product separating in needles, M.P. 151°. After recrystallisation from ether it melted at 153° and gave the following results on micro-combustion (Schoeller): C, 59·39, 59·29; H, 5·57, 5·54; OCH_3, 16·96, 17·01 %; mol. wt., 398, 405; $C_{16}H_{14}O_6(OCH_3)_2$ requires C, 59·32; H, 5·54; OCH_3, 17·04 %; mol. wt., 364·2.

The oxidation product gives no colour with $FeCl_3$ and no precipitate with 2:4-dinitrophenylhydrazine.

0·1192 g. of the oxidation product dissolved in alcohol required 3·50 cc. $N/10$ NaOH for neutralisation to phenolphthalein, corresponding to an equivalent of 361·2 ($C_{18}H_{20}O_8$ titrating as a monobasic acid requires 364·2). On heating with excess of $N/10$ NaOH for 1·5 hours, the equivalent fell to 148·1 and during a further 1·5 hours to 134·7. As this value lies between that required for titration as a dibasic (182·1) and a tribasic (121·4) acid, it seemed probable that a second lactone ring had been formed during oxidation and that this ring on warming with $N/10$ NaOH opened more slowly than the ring already existing in the methyl ether of mycophenolic acid. In order to open the ring completely, 0·6019 g. of the oxidation product was heated with 10 cc. N NaOH for 4 hours in the water-bath. The acid had then used 5·06 cc. N NaOH corresponding to an equivalent of 119. ($C_{18}H_{20}O_8$ titrating as tribasic acid requires 121·4.) The neutralised solution after acidifying and extracting continuously with ether gave an almost quantitative recovery of the unchanged oxidation product, M.P. 153°, not depressed on mixing with that product.

Oxidation under the conditions described with alkaline $KMnO_4$ of the methyl ether of mycophenolic acid ($C_{18}H_{22}O_6$) which titrates in the cold as a mono- and hot as a di-basic acid, thus gives a product $C_{18}H_{20}O_8$ titrating in the cold as a mono- and hot as a tri-basic acid. Oxidation has, therefore, resulted in the formation of a second lactone ring, and this new ring is opened with more difficulty by warming with NaOH than is the original lactone ring present in the methyl ether of mycophenolic acid. The oxidation product also does not immediately decolorise $KMnO_4$ in the cold as does mycophenolic acid and it appears probable therefore that the double bond in mycophenolic acid (see p. 661) has been attacked, resulting in the formation of the new lactonic ring.

The ethereal mother-liquor from the oxidation experiment, on removing the ether and drying, gave a very viscous mass which on rubbing up again with ether gave a second crop, and on repeating the operation, a third and fourth crop of the oxidation product, M.P. 153°. The ether-soluble portion contained a small amount of oxalic acid and a second oxidation product having a much higher methoxyl content, but the amount was too small to obtain it in a pure condition.

In order to obtain this substance in quantity, 10 g. of the methyl ether of mycophenolic acid were dissolved in a slight excess of KOH and brought to the boil. 40 g. of finely powdered $KMnO_4$ were added, 2 g. at a time with shaking. After adding 38 g. the rate of reaction appeared somewhat to decrease. The solution was filtered and cooled, evaporated *in vacuo* to a small bulk, made acid to Congo red and allowed to stand overnight in the cold room. Only inorganic salts separated and the solution was therefore extracted continuously with ether. After removing the ether and drying, 8·5 g. of gum containing a few crystals of oxalic acid remained. This product still contained a considerable amount of the oxidation product, M.P. 153°. It was therefore again dissolved in a slight excess of caustic potash and a further 20 g. of $KMnO_4$ added as before to the boiling solution. The rate of reaction then slowed off very considerably. After filtering, cooling and acidifying nothing crystalline separated and the solution was therefore evaporated *in vacuo* to a small bulk and extracted continuously with ether. The extracted material on drying weighed 8·2 g. It was neutralised, made faintly acid and $CaCl_2$ added. 3·5 g. of dried calcium oxalate were separated.

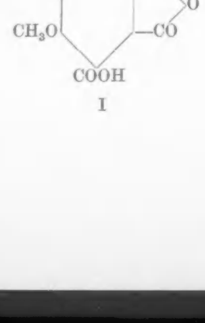
The filtrate after removal of oxalic acid was acidified and extracted continuously with ether, 5·5 g. of a gum remaining after drying over KOH and H_2SO_4. A sample of this material on warming at 70° in a high vacuum readily lost water and set to a white solid which melted at 182°.

The whole of the material was therefore heated in a high vacuum at 70°, the solid so obtained was dissolved in dried ether (10 cc.), dried chloroform (120 cc.) added and the solvent distilled until crystallisation started. After standing overnight, the crystalline material was filtered off and the filtrate evaporated further, yielding a second crop. In this way 1·6 g. of a white crystalline powder were obtained, melting at 210°.

Finally, 1 g. of the above crystalline product was recrystallised by dissolving in a little dry ether, adding dry chloroform and distilling until crystallisation began. In this way 0·48 g. of white crystalline material, M.P. 220° (with evolution of gas) separated, and after drying in a high vacuum gave the following analytical figures (Schoeller): C, 48·10, 48·09; H, 2·67, 2·61; OCH_3, 20·89, 20·91 %. 0·0736 g. on titration in the cold required 9·10 cc. and on heating 9·26 cc. *N*/10 NaOH corresponding to equivalents of 80·9 and 79·5.

$C_{12}H_8O_9$ (Formula I, below) titrating as a tetrabasic acid requires C, 48·64; H, 2·72; OCH_3, 20·95 % and equivalent 74.

Oxidation of the methyl ether of mycophenolic acid $C_{16}H_{16}O_4(OCH_3)_2$ with $KMnO_4$ thus results in the formation first of a dilactonic acid $C_{16}H_{14}O_6(OCH_3)_2$ and then of a compound, which, as will be shown later, must be the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene (I).



I

Presence of a double bond in mycophenolic acid.

Mycophenolic acid reduces permanganate immediately in the cold, and on catalytic reduction with palladium and hydrogen at the ordinary temperature and pressure it absorbs two hydrogen atoms, forming dihydromycophenolic acid. The extreme ease of this reduction makes it very probable that the double bond is aliphatic in nature. Moreover, on determination of the iodine value by the Wijs method, whereas dihydromycophenolic acid does not absorb iodine, mycophenolic acid absorbs two atoms of iodine per molecule.

(*a*) *Dihydromycophenolic acid by reduction of mycophenolic acid with palladium and hydrogen*[1]. 0·5 g. of pure mycophenolic acid, M.P. 141°, was dissolved in 50 cc. alcohol, 0·1 g. of palladium catalyst prepared by heating a solution of palladium chloride with formic acid and 10 % KOH was added and the mixture shaken with hydrogen at the ordinary temperature and pressure for 2 hours. The chief reaction, judged from the absorption of hydrogen, occurred in the first 15 minutes, and no further absorption occurred after 1 hour. The solution was then removed, the catalyst filtered off, water added, the alcohol removed *in vacuo* and the separated crystalline material filtered off and dried. It melted at 139° and this M.P. was unaltered by five recrystallisations from boiling water and aqueous alcohol. The M.P. of a mixture of this substance with mycophenolic acid, M.P. 141°, was 127–130°.

The following figures were obtained by micro-combustion (Schoeller): C, 63·36, 63·54; H, 6·89, 6·82 %; $C_{17}H_{22}O_6$ requires C, 63·30; H, 6·88 %.

0·1585 g. of dihydromycophenolic acid on titrating in alcohol in presence of phenolphthalein gave the first pink colour after addition of 7·75 cc. $N/10$ NaOH. This colour rapidly disappeared and after warming with excess alkali and titrating back, the total amount of alkali used was 9·50 cc., corresponding to an equivalent of 166·9 (theoretical, assumed dibasic, 166). It appears therefore that the acid is an acid-lactone, the lactone ring opening slowly during titration.

(*b*) *Iodine values of mycophenolic and dihydromycophenolic acids.* 0·1588 g. of pure mycophenolic acid and 0·1568 g. dihydromycophenolic acid were allowed to stand in stoppered flasks in the usual way with 10 cc. carbon tetrachloride and 25 cc. Wijs solution along with a third flask containing the same amounts of tetrachloride and reagent and acting as a blank. After standing in the dark for 1½ hours, the contents of the flasks were washed out quantitatively first with 10 cc. 10 % KI solution and then with 300 cc. of distilled water. The solutions were then titrated in the usual way.

The sample of mycophenolic acid used 10·00 cc. $N/10$ iodine (theoretical for absorption of one molecule of I_2 is 9·93 cc. $N/10$ iodine). Dihydromycophenolic acid did not absorb iodine.

Fusion of mycophenolic acid and its methyl ether with KOH.

Fusion of these acids with KOH, at temperatures varying between 210 and 250° for 1 hour, in all cases gave small yields of mixtures of phenols together with tar and mixtures of acids.

In the early experiments the methyl ether of mycophenolic acid (2 g.) was fused with 3 cc. water and 7 g. KOH at 250° for 1 hour, the melt cooled, dissolved in water and the solution saturated with CO_2 and extracted with ether. On removing the ether, 0·2 g. of oily drops remained which set solid in brown radiating needles. This material, after fractionally subliming four times *in vacuo*

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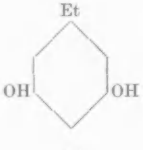
[1] We are indebted to Prof. J. C. Drummond for facilities provided for carrying out this reduction.

(bath temperature 80°) gave 88 mg., of a white product which first showed signs of softening at 97° and melted at 113–115°. This product consisted of white needles which sublimed in the solid state together with a small amount of oily droplets which set solid in radiating needles. It was not possible completely to separate these two products by fractional sublimation. Further sublimation, however, gave 50 mg. of a product which melted at 115–117°.

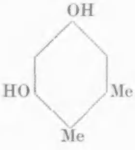
On adding dilute ferric chloride to an alcoholic solution of this product no colour was obtained, though with an aqueous solution a deep blue colour was produced which rapidly disappeared and was replaced by a greyish-white precipitate. With alkalis, the product gave red solutions, and with bleaching powder solution it gave an initial red colour which rapidly faded through reddish-brown to yellow. It did not give the fluorescein reaction with phthalic anhydride, but with sodium hydroxide and chloroform it gave a cherry-red solution with strong green fluorescence (homofluorescein reaction).

The following results were obtained by micro-combustion (Schoeller): C, 70·23; H, 7·59 %; mol. wt., 136. $C_8H_{10}O_2$ requires C, 69·52; H, 7·30 % and mol. wt., 138.

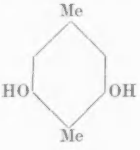
These figures agree moderately well with those required by the formula $C_8H_{10}O_2$, and it appeared probable that the phenol was either an ethyldihydroxybenzene or a dimethyldihydroxybenzene. The colour reactions made it almost certainly an orcinol derivative, and hence the three most probable structures were:



II



III



IV

Of these substances, II, M.P. 93–95°, and IV, M.P. 163°, are unlikely in view of their melting-points and the most probable structure is, therefore, III, 1:5-dihydroxy-3:4-dimethylbenzene (2-methylorcinol). The M.P. of this substance is given by Herzig and Wenzel [1906] as 115–117°, but in a somewhat earlier paper of Simon [1903] as 135°, a figure which was later confirmed by the same author when he obtained it from cetraric acid by treatment with zinc and sodium hydroxide [Simon, 1906]. It was decided, therefore, to prepare the phenol by a diagnostic method, *viz.* the reduction by Clemmensen's method of orcylaldehyde. The product so obtained when freshly prepared melted at 135–136°, and after keeping several months assumed a light brownish tint and melted at 133–135°. The M.P. of our crude fusion phenol (115–117°) was not depressed by admixture with the synthetic phenol. It seemed likely, therefore, that the fusion phenol was 2-methylorcinol, and the fusion technique was altered so as to obtain a purer sample. It was found that by fusing mycophenolic acid itself at lower temperatures (210–220°) a smaller yield of phenol was obtained, but in a much purer condition on fractional sublimation. The fusion was finally carried out as follows.

Mycophenolic acid (8 g.) was fused in 2 g. lots, each with 7 g. KOH and 3 cc. of water in a nickel crucible for 1 hour at 210–220°; the melt was cooled and dissolved in water, the solution was saturated with CO_2 and the phenol fraction extracted with ether. After drying, the phenol fraction weighed 104 mg. This material, on fractionally subliming *in vacuo* with a bath temperature of 75°

gave a solid sublimate, weight, 65 mg., M.P. 129–131°. A second fractional sublimation gave 45 mg. of sublimate, M.P. 130–132°. The mixed M.P. of this fraction with synthetic 2-methylorcinol of M.P. 133–135° was 130–132°. The fraction gave all the colour reactions for 2-methylorcinol. The following results were obtained by micro-combustion (Schoelelr): C, 69·55; H, 7·40 %; mol. wt., 141, 133; OCH_3, negative. $C_8H_{10}O_2$ requires C, 69·52; H, 7·30 %, and mol. wt., 138.

It seemed, therefore, certain that the fusion phenol was 2-methylorcinol, and final confirmation was sought in the preparation of a suitable derivative. Various derivatives were made, using the synthetic phenol, and of these the most suitable proved to be the bis-*p*-bromobenzoate. This derivative cannot be obtained in quantitative yield either by the Schotten-Baumann method, the method of Simon [1903] or by the pyridine method of Einhorn [1898], mixtures of mono- and di-benzoates being obtained. It was obtained in quantitative yield by using the method employed by Lipp and Schoeller [1909] in preparing orcinol dibenzoate.

Synthetic 2-methylorcinol (0·25 g.) was warmed with 1·25 g. *p*-bromobenzoyl chloride for ½ hour at 130°. Hydrogen chloride was rapidly evolved and the reaction appeared complete in 10–15 minutes. The material was cooled and poured into dilute potassium hydroxide. After rubbing with a rod the benzoate readily solidified and was broken up and allowed to stand in KOH solution for 3 days to hydrolyse the excess of bromobenzoyl chloride. The bis-bromobenzoate which was insoluble in KOH was then filtered off, washed with water and recrystallised from alcohol. It melted at 144–145°. The yield was almost quantitative. The following results were obtained by micro-combustion (Schoeller): C, 52·23; H, 3·24; Br, 31·06 %. $C_{22}H_{16}O_4Br_2$ requires C, 52·38; H, 3·18; Br, 31·75 %.

The small amount of fusion phenol above (M.P. 130–132°) was next converted into the bis-*p*-bromobenzoate. It was crystallised from aqueous alcohol, the final product melting at 141–142°.

The M.P. taken simultaneously before and after mixing the fusion and synthetic products were (*a*) synthetic product, 144–145°, (*b*) mixture of synthetic and fusion products, 142–143°, (*c*) fusion product, 141–142°. These results, together with those on the phenol itself, make it certain that the fusion phenol is 1:5-dihydroxy-3:4-dimethylbenzene (2-methylorcinol) III.

Oxidation of mycophenolic acid with concentrated nitric acid.

2 g. mycophenolic acid were dissolved in 25 cc. concentrated HNO_3 and heated in a water-bath at 80° for 1 hour. Excess HNO_3 was then removed by repeated evaporation *in vacuo* and the residual product thoroughly dried over KOH and then over H_2SO_4. The dry residue consisted of a mixture of a yellow oil and crystals of oxalic acid. To remove the latter, the residue was dissolved in water, neutralised and treated with calcium acetate solution. The oxalic acid-free liquid was acidified with H_2SO_4 and continuously extracted with ether. The ether extract after drying and evaporating gave a yellow oil which refused to crystallise (weight, 1·16 g.).

Examination showed that this product consisted chiefly of a lactonic acid and a quantity of this was purified by esterification with diazomethane and fractionation of the esters *in vacuo*. The ester distilled at 100°/0·1 mm. and the fraction from 10 g. mycophenolic acid boiling at 0·1 mm. over a range of 97–103° was collected and weighed (5·2 g.).

The lactonic acid methyl ester gave the following figures on micro-combustion (Schoeller): C, 55·12, 55·16; H, 7·07, 7·04; OCH_3, 18·51, 18·22 %; mol. wt., 216, 210. $C_7H_9O_3(OCH_3)$ requires C, 55·80; H, 7·03; OCH_3, 18·03 %; mol. wt., 172.

The parent acid was then prepared by hydrolysis of the ester by boiling with *N* HCl and extraction of the hydrolysis liquid with ether. On evaporation of the ethereal extract a colourless oil containing no methoxyl remained, which showed no signs of crystallising even after standing *in vacuo* for several months. 0·1969 g. of this dried oil required for titration in the cold 12·3 cc. *N*/10 NaOH, and after heating for ½ hour on the boiling water-bath 24·3 cc. *N*/10 NaOH, corresponding to equivalents of 160 and 81 respectively. $C_7H_{10}O_4$ titrating as a monobasic and dibasic acid respectively requires equivalents of 158 and 79. It is thus obvious that the oxidation acid is a lactonic acid having the empirical formula $C_7H_{10}O_4$, and from its method of preparation and the relative ease with which the lactonic ring opens, it is probably a γ-lactonic acid.

The hydrolysis of the methyl ester with aqueous alcoholic alkali gave a slightly different result and was carried out quantitatively. The ester (1·26 g.) in 25 cc. absolute alcohol was heated in the water-bath for 3 hours with 25 cc. *N* NaOH, and, after cooling and titrating the excess of alkali, had required 14·45 cc. *N* NaOH for neutralisation. ($C_8H_{12}O_4$ titrating as a dibasic acid requires 14·69 cc.) This treatment had, therefore, hydrolysed the ester and opened the lactonic ring. The acid was then isolated by removing the alcohol, acidifying and subjecting to continuous ether extraction. After removing the ether and drying *in vacuo* the acid, although mostly an oil as in the experiment with acid hydrolysis, contained also some well-formed crystals in the form of needles. These were separated by rubbing with a little water and filtering. A further small amount was obtained by drying down the aqueous mother-liquors and repeating the isolation. The total amount isolated represented about 3 % of the ester hydrolysed. After 3 recrystallisations from a small amount of hot water the M.P. was constant at 163°. The following figures were obtained on micro-combustion (Schoeller): C, 53·11; H, 6·50; $C_7H_{10}O_4$ requires C, 53·13; H, 6·36 %.

0·0555 g. required 7·08 cc. *N*/10 NaOH on titration in the cold corresponding to an equivalent of 78·4, and this value was unaltered on treating with excess of NaOH and heating. ($C_7H_{10}O_4$ titrating as a dibasic acid requires an equivalent of 79·0.)

It appears, therefore, that during hydrolysis with alcoholic NaOH a small amount of the lactonic acid has isomerised into a dibasic acid, M.P. 163°. Since teraconic acid ($C_7H_{10}O_4$) is also a dibasic acid and also melts at 163°, a little was synthesised by the method of Petkow [1902] as modified by Stobbe [1903], but the mixed M.P. (depression 20°) showed that our acid was not teraconic acid.

Oxidation of the lactonic acid $C_7H_{10}O_4$ with $KMnO_4$.

Assuming that the lactonic acid $C_7H_{10}O_4$ obtained by acid hydrolysis of the methyl ester contains a γ-lactone ring the position of the three remaining carbon atoms, one of which is present in a carboxyl group, requires to be decided. If they are attached outside the lactone ring to the γ carbon atom, oxidation should give rise to succinic acid. If, on the other hand, the carboxyl group only is attached to the γ carbon, the remaining two carbons must occur as methyl groups attached either both to the α or β carbons or one to each. The latter possibility is ruled out since this would give rise to asymmetric carbon atoms, and hence to optical activity in mycophenolic acid, which is, however, optically inactive. On the basis of the former possibility, oxidation should give rise to $\alpha\alpha'$-dimethylsuccinic acid.

The lactonic acid proved very resistant to oxidation with $KMnO_4$. 1 g. was boiled for 3 hours, 6 hours, 2 and 3 days with $KMnO_4$ (300–500 cc. $N/10$), the hydrated manganese oxide filtered off, the excess $KMnO_4$ destroyed by bisulphite, the fluid evaporated, acidified and subjected to continuous ether extraction after each period of boiling. In each case, however, some unchanged lactonic acid was recovered and the extracted material contained otherwise only oxalic and acetic acids. No succinic or $\alpha\alpha'$-dimethylsuccinic acid was detected.

It would appear, therefore, that the whole molecule is slowly disrupted on oxidation. The constitution of this acid is therefore left unsettled and is being further investigated.

DISCUSSION AND SUMMARY.

Mycophenolic acid has been shown to have the empirical formula $C_{17}H_{20}O_6$. The molecule contains the following groupings.

(*a*) A OCH_3 group, which can be almost quantitatively removed by heating with HI at 85°, thus yielding *nor*mycophenolic acid, $C_{16}H_{18}O_6$.

(*b*) A phenolic OH group which can be methylated and acetylated and is responsible for the blue $FeCl_3$ colour.

(*c*) One COOH group which titrates quantitatively in the cold and can be esterified and regenerated by hydrolysis of the ester.

(*d*) A lactonic ring $\underset{\diagdown\mathrm{O}\diagup}{\mathrm{CO}\text{----}\mathrm{C}}$ which titrates almost quantitatively in the cold in mycophenolic acid, but which titrates only on heating in the methyl ether and the acetyl derivative of mycophenolic acid.

(*e*) The grouping $>\!C{=}C\!<$ which readily adds two atoms of iodine, and is readily reduced at ordinary temperatures and pressures with palladium and hydrogen, giving dihydromycophenolic acid, $C_{17}H_{22}O_6$, and hence is probably aliphatic in nature.

The following facts have also been demonstrated.

(*a*) Mycophenolic acid is optically inactive.

(*b*) On fusion with KOH, mycophenolic acid gives 1:5-dihydroxy-3:4-dimethylbenzene (2-methylorcinol). It is the same methylorcinol as was obtained by Simon [1903] from the lichen acid cetraric acid and, together with the other metabolic products, of *P. brevi-compactum* and related species affords the first examples correlating mould metabolic products with lichen acids. All these mould metabolic products are also closely related structurally to the lichen acid divaricatic acid, the constitution of which has been determined by Asahina and Hirakata [1932].

(*c*) On oxidising with $KMnO_4$, the methyl ether of mycophenolic acid $C_{18}H_{22}O_6$ gives rise to a product $C_{18}H_{20}O_8$ which is not ketonic in nature since it gives no precipitate with 2:4-dinitrophenylhydrazine. This substance titrates in the cold as a monobasic acid and on heating as a tribasic acid, indicating the presence of two lactonic rings. Of these the newly formed ring opens on heating with alkali with much greater difficulty than that originally present in the molecule.

(*d*) On further oxidation with alkaline $KMnO_4$ the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene was obtained. The formula I (p. 660) of this substance is evident for the following reasons.

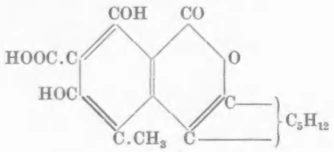
(i) The methoxy groups must be in the 1:5 positions since they are in these positions in the phenol (2-methylorcinol) obtained on potash fusion of mycophenolic acid.

(ii) The carboxyl groups must then occupy the 2:3:4:6 positions since the substance is the anhydride of a tetracarboxylic acid.

(iii) Because of the symmetry of the molecule only one mono-anhydride is possible and this must have formula I.

(*e*) On oxidising with HNO_3, mycophenolic acid gives a quantitative yield of a lactonic acid $C_7H_{10}O_4$. This acid contains a COOH group and a lactone ring and is an uncrystallisable oil, which on warming with alcoholic NaOH partly isomerises, giving a dibasic acid $C_7H_{10}O_4$, M.P. 163°, which is not identical with teraconic acid (M.P. 163°). The lactonic acid is very resistant to oxidation with $KMnO_4$ and gives rise to oxalic and acetic acids but no succinic or dimethylsuccinic acid could be detected.

On the basis of these facts, the following structure for *nor*mycophenolic acid, $C_{16}H_{18}O_6$, is tentatively advanced:



V

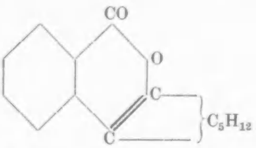
in which the constitution of the aliphatic residue remains to be determined. Mycophenolic acid then becomes the monomethyl ether of this substance, but there is at present no conclusive evidence as to which phenolic group is methylated. The constitution of the nitric acid oxidation product $C_7H_{10}O_4$ is being further investigated in order to settle the remainder of the structure of *nor*-mycophenolic acid.

The formula accounts for the following properties of mycophenolic acid.

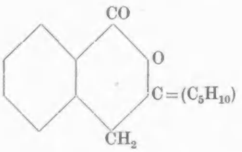
(*a*) Its optical inactivity.

(*b*) The very intense and permanent blue colour given by both mycophenolic and *nor*mycophenolic acid with $FeCl_3$. Thus the arrangement of the OH and COOH groups in the benzene ring characterises the substance as a derivative of γ-resorcylic acid. Known derivatives of this acid, *e.g.* *p*-orsellinic (4-methyl-2:6-dihydroxybenzoic) acid and *m*-xylorcinolcarboxylic (3:5-dimethyl-2:6-dihydroxybenzoic) acid give the same intense stable blue colour. Also 1:5-dihydroxybenzene-3:6-dicarboxylic acid (β-resodicarboxylic acid) gives a colour in alcoholic solution with $FeCl_3$ which is indistinguishable from that of mycophenolic acid.

(*c*) The presence and position of an aliphatic double bond. The double bond, if the compound is to be optically inactive, must be placed either in or immediately outside the lactone ring and can have one of only two positions:



or



Of these the former is preferred as it explains most readily the formation of the dibasic acid $C_7H_{10}O_4$ on oxidation with HNO_3, the second formula leading only to a monobasic acid.

(*d*) The formation of 2-methylorcinol from mycophenolic acid by KOH fusion.

(*e*) The formation from the methyl ether of mycophenolic acid by $KMnO_4$ oxidation of the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene.

(*f*) The relation of mycophenolic acid to other products of *P. brevi-compactum* and related species. It has already been shown [Clutterbuck, Oxford, Raistrick and Smith, 1932] that in addition to mycophenolic acid, *P. brevi-compactum* and related species produce a series of other acids having the empirical formulae $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$, $C_{10}H_{10}O_7$ and $C_8H_6O_6$. The last acid has been characterised as 3:5-dihydroxyphthalic acid [Oxford and Raistrick, 1932] whilst the acids $C_{10}H_{10}O_5$, $C_{10}H_{10}O_6$ and $C_{10}H_{10}O_7$ have been shown to be interconvertible and to be respectively 3:5-dihydroxy-2-carboxybenzylmethylketone, 3:5-dihydroxy-2-carboxyphenylacetylcarbinol and a hydrated form of 3:5-dihydroxy-2-carboxybenzoylmethylketone [Oxford and Raistrick, 1933]. The relation of these four acids to mycophenolic acid is obvious.

We wish to express our gratitude to Prof. Robert Robinson for much kindly advice and criticism given during the work described in this and the previous paper.

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LXXXVII. SULLIVAN'S REACTION FOR THE QUANTITATIVE DETERMINATION OF CYSTEINE AND CYSTINE.

BY JOSEPH W. H. LUGG.

From the Division of Animal Nutrition of the Commonwealth Council for Scientific and Industrial Research, University of Adelaide, South Australia.

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SULLIVAN [1926] has adduced reasons for believing that his colour test for cysteine, depending upon the production of a red-coloured soluble complex of cysteine and sodium 1 : 2-naphthoquinone-4-sulphonate, is specific for cysteine among the sulphydryl compounds. Various substances form coloured complexes with the quinone, but Sullivan found that in the presence of cyanide under conditions referred to later, the complex with cysteine was the only one that would withstand subsequent treatment with $Na_2S_2O_4$.

In later communications Sullivan [1929, 1, 2] develops his methods, explains his preference for the "sulphite modification," and briefly mentions the effects of the buffering capacity of a hydrolysate.

Sullivan's procedure for the estimation of cysteine consists essentially in allowing the cysteine to react with the reagent in alkaline solution. Subsequently sulphite and more alkali are added and are followed after the lapse of from 10 to 30 minutes by an alkaline solution of sodium hydrosulphite. After the addition of the $Na_2S_2O_4$ the red colour due to cysteine is retained whilst colours due to other amino-acids are changed to yellow. Sullivan advocates addition of a small quantity of cyanide before the reagent "to check oxidation" of the cysteine, and states that the addition of a little more cyanide after the hydrosulphite will "stabilise" the colour. The procedure for cystine consists in first treating the solution with plenty of cyanide and then proceeding as for cysteine. Sullivan believed that the cyanide "reduced" the cystine to cysteine to a varying degree depending upon the amount of cystine present, and that a cysteine unknown must therefore be compared with a cysteine standard similarly prepared, and a cystine unknown with a cystine standard.

Sullivan's procedure provides a valuable colour test for cysteine, but experience of his quantitative methods in this laboratory has not been satisfactory. Thus it was found: (1) The depth of red colour is variable and far from proportional to the amount of cysteine or cystine present. Rimington [1929] had already noted this poor proportionality and advised having the unknown within 15 % of the standard in an estimation. (2) Even when compared with a standard of nearly the same colour, the results for the unknown are often irregular. (3) The presence of relatively considerable amounts of other amino-acids diminishes the amount of red colour due to cysteine or cystine, and the yellow colour due to the other amino-acids interferes when a pure cysteine or cystine standard is used for comparison. The diminution in red colour was found to be attributable in part to the buffering effects of the amino-acids present upon the p_H of the solution[1].

Two directions in which the accuracy of the method might be improved suggested themselves: (1) to maintain the same p_H in standard and assay, and (2) to swamp both standard and assay with a considerable amount of amino-acid.

It was found possible to effect both objects by adding glycine with appropriate amounts of alkali, and by this means, two of the most serious drawbacks to Sullivan's procedure (unreliability and lack of proportionality) were overcome.

It was found that the reaction between the cystine and cyanide was the double decomposition:

$$RSSR + NaCN \longrightarrow RSNa + RSCN$$

discovered by Pulewka and Winzer [1928]. Under conditions that may be varied considerably, this reaction proceeds so nearly to completion that any deviation from it cannot be detected colorimetrically.

Reagents required.

A. N NaOH solution.

B. M Glycine buffer in 0·4 M tertiary sodium citrate[2]. 7·50 g. of glycine and 14·3 g. of the salt $C_6H_5O_7Na_3$, 5·5 H_2O, are dissolved in 50 cc. of water + 25 cc. of solution A and diluted to 100 cc.

C. M NaCN solution in 0·1 N NaOH solution.

D. 0·02 M Solution of sodium 1 : 2-naphthoquinone-4-sulphonate.

E. 0·5 M Na_2SO_3 solution in 0·5 N NaOH solution.

F. 2·5 N NaOH solution.

G. 0·1 M $Na_2S_2O_4$ solution in 0·5 N NaOH solution.

Solutions B and C may not be mixed and stored as one, because a slow reaction proceeds, leading to the development of a brown colour not removed by $Na_2S_2O_4$. Solutions A, B, E and F may be stored for months in well-stoppered bottles. Solution C may be kept for several weeks. Solutions D and G must be prepared immediately before they are required.

THE DETERMINATION OF CYSTEINE AND CYSTINE IN PURE SOLUTIONS[3].

(a) *When either cysteine or cystine alone is present.*

An aliquot (a cc.) of the unknown solution containing preferably between 1 and 2 mg. of cystine or 0·5 and 1 mg. of cysteine, is titrated with b cc. solution A (or F if necessary) to p_H 9·5 using thymol blue indicator. This mixture is titrated with solution F (c cc.) to about p_H 12·5 using orange G.G. (alizarin yellow R is hardly satisfactory because its range does not extend much above p_H 12·0).

5 cc. of solution B and 2 cc. of solution C are transferred to a 25 cc. stoppered cylinder; b cc. of solution A (or F, if used) are added and followed by $5 - a - b$ cc. of water. The contents are mixed and a cc. of the unknown solution are added. The contents are thoroughly mixed and are then allowed to react for 10 minutes. Solution D is made up during this interval and 1 cc. of the fresh solution D is added to the contents of the cylinder, which are again mixed. After a further interval of 5 minutes, 5 cc. of solution E are added and mixed and are followed

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[1] The work recorded in this article was performed at temperatures in the vicinity of 25°.

[2] The presence of citrate in solution B is desirable when calcium salts occur in the unknown. The amount may be increased or diminished if desired.

[3] Various steps in the procedures are explained in the discussion.

by $1 - c$ cc. of water and then by $5 + c$ cc. of solution F. The cylinder's contents are again thoroughly mixed and allowed to react for 20 minutes. 5 minutes before this period is completed, solution G is prepared and at the conclusion of the 20-minute interval, 1 cc. of solution G is added, and the contents of the cylinder are again mixed. After standing for 10 minutes, the solution is ready for colorimetric comparison with a cystine standard similarly prepared at the same time and between 0·7 and 1·5 times as intense in colour. The maximum safe red colour intensity is that obtained with 3 mg. of cystine. Solutions must be allowed to stand in the colorimeter cups with plungers immersed approximately to the right depths for at least 3 minutes before final readings are taken, and these should be completed within 30 or 40 minutes of adding solution G. With the standard containing s mg. of cystine and set at y mm. in the colorimeter and with the unknown matching at x mm., the unknown will contain sy/x mg. of cystine, or $121sy/240x$ mg. of cysteine.

The above procedure has been found satisfactory for estimating cysteine or cystine in pure solution to within 1 or 2 %. The final colour solutions which ordinarily have a volume of 25 cc. cannot safely be diluted to a different volume because of the risk of oxidation. For this reason, the different reagents should be pipetted accurately into the cylinders.

(*b*) *When both cystine and cysteine are present.*

Except by recourse to the somewhat unsatisfactory procedure of blowing air through a mixed cysteine-cystine solution first rendered slightly alkaline at p_H 7·5–8, to oxidise the cysteine present, it has not been found possible to estimate the two amino-acids when present together. If the red colour from an aliquot of the unoxidised solution corresponds with u mg. of cystine, and the colour from an equivalent aliquot after aerial oxidation corresponds with v mg., then the actual amounts present are $(u - v)$ 121/120 mg. of cysteine and $2v - u$ mg. of cystine.

DETERMINATION OF CYSTEINE AND CYSTINE IN PRESENCE OF OTHER SUBSTANCES.

The methods are in every way the same as those already given for the determinations in pure solutions. Errors may arise from two causes. (1) Substances present in the unknown solution may interfere with the colour reaction. (2) The initial colour of the unknown solution may contribute towards the final colour to be read.

Sullivan has dealt very thoroughly with the question of specificity. He claims that the cysteine colour reaction is most probably confined to compounds containing the group $—\overset{|}{C}(SH)—\overset{|}{C}(NH_2)—COOH$.

His conclusions concerning the interference of iron salts may be amplified somewhat. Ferric salts may interfere badly in the estimation of cystine. If added before the cystine and allowed to stand with the buffer and cyanide, they may cause only slight loss of colour. If added after the cystine, however, the loss may be very serious. In the table of results given below, this contrast in effects is very striking. Unfortunately, it has not been found possible to eliminate these effects and on this score there will always be some degree of uncertainty in reporting the cystine contents of solutions containing unknown amounts of ferric salts. With ordinary tissue hydrolysates, this error will be unlikely to exceed 1 or 2 %. Ferric salts form a complex with cysteine which is later liberated in the oxidised form whilst the iron appears in the reduced form. The cystine so formed can then react with more cyanide to form cysteine and

RSCN, and these processes will continue with gradual conversion of the cysteine or cystine into RSCN until all the ferric iron is reduced or all the cystine is converted into RSCN. This is probably the explanation of the effects mentioned above. The iron could also act as a carrier between the cysteine and atmospheric oxygen, if present.

In so far as many heavy metal salts are unlikely to be present in solutions to be tested, their effects have not been investigated in detail. Had it been found that zinc salts would not interfere, a simpler procedure depending upon reduction by metallic zinc might have been evolved for the estimations of cysteine and cystine when present together. One-tenth of a millimol of $ZnSO_4$ added after the cyanide and before the cystine caused a loss of about 20 % in colour and a rapid fading later.

Errors of type (2) may sometimes be eliminated by colorimetric compensation. The colour of a hydrolysate due to humin may sometimes be removed by adsorbents without appreciable loss of cystine, if particular precautions are taken. The brown humin colour, contrary to what might be expected, is not appreciably bleached in any stage of the colour reaction. Blanks for use in compensating colorimeters may be prepared if required. One is made from the standard cystine solution and one from the unknown by introducing 0·1 cc. of M $FeCl_3$ solution 5 minutes before adding the quinone reagent. Alternatively, the blanks may be made to contain no iron by omitting the quinone reagent and substituting pure water.

Tissue hydrolysates may sometimes give slightly cloudy colour solutions, particularly following the addition of the hydrosulphite. It is essential that the solutions be crystal clear for accurate comparison, and in such cases the colour solutions (standard as well as cloudy unknown) should be filtered through rapid paper with the least possible agitation before comparison.

ANALYTICAL RECOVERY OF CYSTINE FROM ADDED SUBSTANCES.

A gelatin and a gelatin *plus* carbohydrate hydrolysate were prepared by hydrolysing 5 g. of acid-washed gelatin, and 5 g. of acid-washed gelatin *plus* 1 g. of filter-paper cellulose *plus* 1 g. of sucrose, separately each with 50 cc. of $5N$ HCl solution. After remaining for 24 hours at the boiling-point the solutions were filtered, evaporated to syrups upon the water-bath, diluted to 15 cc. with water and brought to p_H 2·5 (spot indicator) with M tertiary sodium citrate solution (about 25 cc.). At this p_H the humins generally are least soluble. After standing for 48 hours, the solutions were filtered and diluted each to 50 cc. These solutions constitute the A 1 (gelatin only) and B 1 (gelatin *plus* carbohydrate) referred to in Table I. From each of these solutions, 25 cc. were taken and treated with 1 g. of acid-washed kieselguhr and then after filtration with two successive portions each of 0·5 g. of acid-washed fine norite charcoal. The solutions were filtered after each treatment with norite and constitute finally the A 2 and B 2 of Table I. Neither cysteine nor cystine could be detected in A 2 or B 2. A 1 and more particularly B 1 did not give pure yellow blanks in the cysteine and cystine tests, but the amount of brown colour was very small and presumably owed its origin entirely to the initial brown colour of the solutions. The initial brown colours of A 1, B 1, A 2 and B 2 have been compared colorimetrically with a fresh ferric ferricyanide solution made by adding 5 cc. of $M/20$ $FeCl_3$ solution to 5 cc. of $M/30$ $K_3Fe(CN)_6$ solution. Matchings were not perfect by any means. The brown colour intensities in terms of this standard were approximately 0·5, 1·2, 0·01, and 0·08 respectively.

Table I.

Extraneous material added	Cystine present mg.	Cystine estimated mg.	% recovery
1 cc. A 2. Just before the cystine	2·00	2·03	101·5
1 cc. A 2. ,,	1·00	1·01	101·0
2 cc. A 2. ,,	1·00	1·025	102·5
1 cc. B 2. ,,	2·00	2·00	100·0
1 cc. A 1. ,,	2·00	1·98	99·0
1 cc. B 1. ,,	2·00	1·93	96·5
1 cc. B 1. ,,	1·00	0·955	95·5
100 mg. arginine. Just before the cystine	1·05	1·07	102·0
10 mg. tyrosine. ,,	1·00	1·015	101·5
1/300 millimol $FeCl_3$. 5 min. before the cystine	2·00	1·83	91·5
1/150 ,, ,,	2·00	1·68	84·0
1/30 ,, ,,	2·00	1·15	57·5
1/150 ,, 5 min. after the cystine	2·00	0·53	26·5
1/30 ,, ,,	2·00	Trace	?
1/12 ,, 5 min. after the cysteine	(1·00)	(0)	0

The effects of varying amounts of these hydrolysates and of a few pure amino-acids upon the estimation of cystine are recorded in Table I. Readings were made with the aid of an ordinary Klett (non-compensating) colorimeter. The results demonstrate the applicability of this method to the estimation of cystine in hydrolysates, when the cystine amounts to approximately 2, 1, or even 0·5 % of the amino-acids present, and when the decomposition products of a considerable amount of carbohydrate are present also. At the same time, they reveal a tendency to underestimate slightly the cystine present when humin substances occur in a solution. Mercaptans generally condense with aldehydes and ketones and the loss might be accounted for on these grounds. Clearly it is not always essential to decolorise a hydrolysate or to employ a compensating instrument with appropriate blanks.

DISCUSSION.

During the reaction between the cystine and cyanide, the p_H of the solution is approximately 9·5 in the procedure given, and colour development in the final period takes place at about p_H 12·5. At this higher p_H cysteine undergoes some decomposition, but if the p_H is lowered by only 0·5 unit the amount of red colour is diminished 20–30 %. Further, if the reaction between the cystine and cyanide takes place at p_H 12·5, little colour develops on subsequently adding the quinone and other reagents, and what develops fades rapidly. For these reasons it is necessary to control the p_H in the two regions, such control being achieved with the aid of the glycine buffer.

If the amount of cyanide is halved, the red colour due to 1 mg. of cystine is diminished by about 25 %; if doubled or trebled the final colour solutions become brown transiently upon slight agitation in air. The canary-yellow colour obtained in the absence of cysteine and cystine varies more or less directly with the amount of quinone present. If the quinone be reduced to half, the loss of red colour developed by cystine is not appreciable unless more than 3 mg. of cystine are present. On the other hand, the colour developed by cystine is unaccountably reduced if the amount of quinone be much increased.

In consequence of this anomalous behaviour, an attempt to derive the stoichiometric relation between cysteine and the quinone colorimetrically could not lead to definite results, the more so because of the recognised instability of the quinone. The indications from colour readings with varying excesses of cysteine suggested that two molecules of cysteine react with one of quinone.

Employing Sullivan's procedure with 1 mg. (1/240 millimol) of cystine the red colours obtained with (approximately) 1/50 millimol of the quinone are of the same order, whether 1/5 millimol of mixed amino-acids are present or not. As all these amino-acids can react with the quinone, one is led to conclude either that the group or groups involved in the reaction with cysteine are not the same as those involved in the reaction with other amino-acids, or failing that, that the cysteine complex will always be formed at the expense of any other. The first alternative is supported by the known influence of $Na_2S_2O_4$ upon the colours produced. Amino-acids almost certainly react at the sulphonate group in the reagent in the ordinary amino-acid reaction [but see Folin, 1922], leaving the carbonyl groups for reduction by $Na_2S_2O_4$ in alkaline solution or by sulphite at p_H 6–7, whereas presumably, the cysteine reacts in some way with the carbonyl groups which cannot then be attacked readily by $Na_2S_2O_4$ or acid sulphite.

The colour solutions bleach slowly on standing in absence of air and turn brown (transiently if excess $Na_2S_2O_4$ is present) when agitated with air. In the early stages, the fading is sensibly proportional to the cysteine or cystine content and, therefore, does not lead to errors in comparison. The amount of hydrosulphite may be increased to check aerial oxidation with consequent browning, but only at the expense of increased fading of the red colour from cysteine.

Both sulphite and cyanide react with cystine in such manner that for each molecule of cystine, one molecule of cysteine and one of something else are produced[1]. It would, therefore, appear that sulphite or cyanide alone would suffice for the colour reaction. With sulphite alone, very little colour develops, whereas with cyanide alone the full colour is obtained with pure cystine solutions, and addition of sulphite, as in the procedure given, may be regarded as a precautionary measure against adventitious oxidation.

SUMMARY.

Sullivan's colour test for cysteine has been examined with the object of improving his procedure for estimating cysteine and cystine in solutions containing these amino-acids. A method has been evolved which permits the colorimetric estimation of cysteine or cystine (to within 1 or 2 %) in pure solution. The same method has been applied successfully to the estimation of cystine in the presence of large quantities of other amino-acids and of the hydrolysis products of carbohydrates. Some of the possible sources of error have been discussed.

I wish to record my indebtedness to Sir C. J. Martin, at whose suggestion this work was done, for his notes on earlier experiments with Sullivan's reaction and for his criticisms and assistance.

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[1] In a previous communication [Lugg, 1932], I discussed the reaction with sulphite. At the time it was submitted for publication, I was not aware that Clarke [1932] had arrived at the same conclusions.

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LXXXVIII. A SPECTROGRAPHIC ANALYSIS OF THE METALLIC CONTENT OF MECONIUM.

By JOSEPH HAROLD SHELDON
(from the Royal Hospital, Wolverhampton)
and HUGH RAMAGE
(Ridgemont, Carrow Hill, Norwich).

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In the course of an investigation into the metallic content of the liver in foetal and infant life, four specimens of meconium were examined. The results were of interest in that they appeared to show that while copper and manganese are excreted in considerable quantities into the bowel during foetal life, iron appears to be almost entirely retained in the body of the foetus. These results were consistent with those obtained by examining the foetal gall-bladder, which contains in particular a notable amount of manganese, this being the only metal which reaches a greater concentration in the foetal gall-bladder than in the foetal liver, and the fact that the spectrogram of meconium is chiefly remarkable for the strength of the manganese lines suggested that there is a continual excretion of this metal by way of the bile during foetal life. These findings were of sufficient interest to merit a more extended survey, the results of which are given in this paper. Twenty-four specimens of meconium were examined, derived from twenty-four healthy full-term infants born at the Maternity Department of the Royal Hospital, Wolverhampton. The first specimen of meconium passed by each infant was that used for analysis. Owing to the extent to which the meconium adheres to the linen, and to the difficulty of separating the faeces formed during intra-uterine life from those formed immediately after feeding had begun, it was not possible to obtain any quantitative results as to the total amount of each metal that had been excreted during the period of intra-uterine life. The investigation has this interest, that it not only helps to elucidate the nature of the metallic interchange between mother and foetus, but it may also give some indication whether, under the conditions of modern life, there is any marked deficiency in the maternal supply of those many metals, which as we now know are necessary to life.

The meconium was separated from the napkin, dried to constant weight at 100° and burnt before the slit of a quartz spectrograph by the method of Ramage [1929]. Quantitative estimations were made by comparing the intensities of the lines so obtained with those derived from burning papers with varying amounts of the standard solution. A specimen of the type of linen napkin was also burnt. This gave weak lines due to Na and K and a strong Ca line, but was otherwise negative. The following table shows the results so obtained for Cu, Mn, Fe, Ca, Mg. (In all cases the percentage of dry weight is indicated.)

Table I.

	Percentage of dry weight					
No.	Copper	Manganese	Iron	Calcium	Magnesium	Silver
1	0·008	0·004	−0·02	0·30	0·24	—
2	0·01	0·002	−0·02	1·05	+ +0·48	—
3	0·01	0·0065	−0·02	0·27	0·12	—
4	0·01	0·003	−0·02	0·27	0·18	—
5	0·02	0·007	−0·02	+1·44	0·12	—
6	0·02	0·003	0·025	0·30	0·30	Slight trace
7	0·007	0·007	−0·02	0·18	0·24	—
8	0·012	0·008	0·03	1·05	+0·48	Slight trace
9	0·02	0·006	−0·02	0·30	0·15	—
10	+0·02	0·006	0·13	0·18	0·09	—
11	0·02	0·0023	−0·02	0·15	0·12	—
12	0·018	+0·008	0·04	0·24	0·24	—
13	0·02	0·002	−0·02	0·21	0·21	—
14	0·01	0·008	0·02	0·21	0·21	—
15	0·02	0·005	−0·02	0·36	0·36	—
16	0·008	0·005	0·02	0·21	0·21	—
17	0·006	0·005	−0·02	0·18	0·18	—
18	0·017	0·006	0·02	0·14	0·12	Trace
19	0·02	0·007	0·02	0·30	0·18	—
20	0·019	0·002	−0·02	0·24	0·12	Slight trace
21	0·019	0·008	−0·02	0·36	0·24	—
22	0·019	0·006	−0·02	0·27	0·09	—
23	0·018	0·003	0·03	0·15	0·18	—
24	0·019	0·003	0·02	0·24	0·24	—

Copper. This metal was present in every specimen, usually in quite considerable amounts. The amount varied between 0·006 % and slightly in excess of 0·02 % (measured by the 3274·0 and 3247·5 lines). The bulk of the figures, however, were at the higher end of the scale, fifteen of the twenty-four specimens having a percentage of copper in excess of 0·017. There is therefore a definite excretion of copper by way of the bowel during intra-uterine life. From our previous investigations on the foetal gall-bladder, it is probable that this occurs by way of the bile. Since all the infants were healthy it appears justifiable to assume that the amount of copper supplied by the mothers was more than sufficient to provide for the storage of this metal in the liver which occurs during foetal life. This suggests that with an ordinary working class diet the mother succeeds in providing a sufficient supply of copper for this purpose, though it is possible that the provision of this supply may, in the absence of a sufficient diet, be detrimental to the mother. (It is assumed that any mechanism concerned in the fixing of copper in the foetal tissues was working normally.)

Manganese. The actual percentage of manganese in meconium is much less than is the case with the other elements, but it must be remembered that in the normal body this metal only occurs in minute traces (about 0·0008 % of the dry substance). The spectrogram of meconium is, however, quite characteristic, and differs from that of all the other tissues we have examined in the intensity of the manganese triplet (4030·8; 4033·1; and 4034·5). It will be seen that this metal is found in amounts ranging from 0·002 % to slightly in excess of 0·008 %. The cases are equally distributed throughout this range. There are four cases which reach the maximum figure of 0·008 %. The significance of these figures may be realised from the fact that in an examination of some 200 adult tissues [Sheldon and Ramage, 1931] the highest figure found, and then only exceptionally, was 0·002 %. It is obvious therefore that the mother supplies the foetus with a great excess of manganese. The reasons for this are obscure. It must be remembered that no foetal store of this metal is built up in the liver as is the case with copper and iron. The organism appears to maintain the concentration within fairly close limits at all ages, and it would appear that any excess is excreted. It is, however, possible that while manganese is not stored in the foetus, a constant supply is necessary for foetal growth, which is again steadily excreted. From the high content in the gall-bladder of the foetus of this metal, it would appear that the path of excretion is, as with copper, *via* the bile. The figures certainly do not suggest that, in the cases examined, the mother had any difficulty in supplying the foetus with all the manganese it required.

Iron. Iron differs from all the other metals present in meconia in that while with them there was no difficulty in matching the various lines with varying strengths of the standard solution, the iron lines barely reached the intensity of those in the weakest standard used. These standards were originally prepared so as to cover the amounts of the different metals that would, in the quantities burnt, be found in the tissues of the body. In a general examination of the body it was very rare for the iron lines to be too weak for comparison with the least amount of standard employed [for details see Sheldon and Ramage, 1931]. In the case of meconium, however, no less than fourteen specimens (58 %) showed iron lines that were too weak for estimation by this method. In many cases, indeed, the iron lines were only just visible, indicating that only the merest traces of this element were present in the meconium. Iron therefore provides a sharp contrast with the other metals that have just been considered. This may be brought out in another way. The average amount of copper in the human liver at full-term is 0·03 % (dry), and many of the meconium specimens approach this figure. The corresponding figure for iron is 0·25 % (dry). None of the meconium figures approaches this, the highest being 0·13 %, and most of the cases with identifiable amounts are about 0·02 %, or a tenth of the liver figure at birth. Indeed the weakness of the iron lines is as striking a feature of the spectrograms of meconia as is the strength of the manganese, and this investigation entirely bears out the provisional results which led to it. This poorness in iron may be due to several causes. (1) It may be that the avidity of the foetal tissues for iron, and especially of the liver, is such that all the iron received from the mother, however much, is retained. Foetal gall-bladder and foetal bile contain iron in considerable amount, which suggests that during foetal life the metal is excreted by this route, and its absence from the meconium in similar amounts must be ascribed to a reabsorption from the bowel, which is in favour of a marked avidity of the foetal tissues for the metal. The human liver in infancy and at birth shows great variations, however, in its iron content, and it would have been thought that this variation would be reflected in the meconium. (2) An equally possible suggestion is that while the diet of the pregnant mothers in this midland district furnishes adequate quantities of copper and manganese, it is deficient in iron, so that the foetus is compelled to retain all that it receives in order to build up the necessary reserve store. It is probable that both factors are operative, but the problem merits further enquiry.

Magnesium. The bands of this element centred near wave lengths 3820 and 3684 are clearly visible in all the spectra. The metal occurred in amounts ranging from 0·09 % to a figure in excess of 0·48 %. Cases 2 and 8 were exceptional in the amount of magnesium they contained, which was too strong for measurement by this method. Of the twenty-four cases, twenty had figures between 0·09 and 0·24 %. The excretion of magnesium during foetal life appears to be remarkably uniform.

Calcium. All the meconia contained appreciable amounts of calcium, though there was a considerable degree of individual variation. The figures ranged between 0·15 % and the enormous figure of approximately 1·44 %. Whether cases 2 and 5 are due to an increased excretion of calcium by the foetus as a result of some derangement of their calcium metabolism is obscure, but it would appear more probable that in both cases the large amounts found represent an increased supply from the mother. (The results with Ca are, however, in some doubt, since, although every effort was made to obtain specimens free from linen fibres, the spectrogram of the napkins showed that they contained considerable amounts of calcium.)

Sodium and potassium were found in all the specimens. The strength of the sodium lines was remarkably uniform, but the potassium showed a greater degree of individual variation. Traces of rubidium were found in nearly all the specimens. Traces of silver were identified in cases 6, 8, 18, 20.

SUMMARY.

The examination of the first meconium from twenty-four apparently healthy babies gave the following results.

1. Lines due to Na, K, Ca, Mg, Cu, Mn, Fe are recognisable in all. (It is difficult to determine the presence of Zn by this method, and no opinion is offered with regard to this metal.)

2. The most remarkable features of the spectrograms are the strength of the lines due to manganese (and to a less extent, those due to copper) and the weakness of the lines due to iron. The reason for this constant over-supply of manganese to the foetus is obscure.

3. The results suggest that, at any rate under the social conditions of town working class life, the mother is able to supply the foetus with an excess of all metals except iron. Either, however, she is not able to do so in the case of iron, or the avidity of the foetal tissues for this metal is so great that almost all the iron received from the mother is retained, and any excreted into the gut is reabsorbed.

4. The quantities of the metals found indicate considerable variations in the mothers' power to supply them. One case, however, No. 8, shows a high percentage and another, No. 17, a low percentage common to all metals: such general cases are rare.

Our thanks are due to Miss Humphries, Sister-in-Charge of the Maternity Department at the Royal Hospital, Wolverhampton, for her help in obtaining specimens, and to Dr S. C. Dyke, Hon. Pathologist to the Hospital, for extending Laboratory facilities.

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LXXXIX. THE REDUCTION POTENTIALS OF CYSTEINE, GLUTATHIONE AND GLYCYLCYSTEINE.

By DAVID EZRA GREEN.

From the Biochemical Laboratory, Cambridge.

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Introduction.

Dixon and Quastel [1923] investigated the potentials of cysteine-cystine and glutathione-oxidised glutathione mixtures. They observed that these two systems presented peculiar difficulties, in that the potentials exerted at noble metal electrodes were drifting and difficult to reproduce. The platinum-calomel cell usually employed for the determination of the reduction potentials of the type quinol-quinone was found to be unsuitable for work on the RSH—RSSR type, but with the use of the gold-calomel cell satisfactory results were obtained. Unlike the potentials of typically reversible systems, both the cysteine and glutathione potentials depended only upon the p_H and the logarithm of the concentration of reduced component. The oxidants in both cases did not affect the electrode. The relationship of the variables in the electrode equation was shown to be the following:

$$E_h = E_0 - \frac{RT}{F} p_H - \frac{RT}{F} \log RSH.$$

Michaelis and Flexner [1928] showed the above equation to hold for cysteine solutions, regardless of the nature of the electrode employed with the exception of the solid gold electrode. By carefully excluding all traces of oxygen from their cell, they obtained identical potentials at electrodes of blank platinum, gold-plated platinum and mercury, reproducible to within 5 millivolts. The most rapid and consistent results came with the use of the mercury electrode. An E_0 of $-0{\cdot}001$ volt was assigned to the electrode equation.

Kendall and Nord [1926] claimed that if an activating agent like indigo carmine was added to a mixture of cysteine and cystine, the reduction potential was determined by the ratio of RSH to RSSR. Their evidence, however, is fragmentary and has been disputed by Dixon and Tunnicliffe [1923] and by Michaelis [1929]. Williams and Drissen [1930] oxidised cysteine to cystine by oxidising agents (I, KIO_3, $K_2Cr_2O_7$, *etc.*) and attempts were made to locate the redox potential by electrometric titration. When so oxidised by titration methods, the cysteine system showed a variation of potential as the oxidation proceeded, which resembled that of the ordinary reversible oxidation-reduction system. However, Ghosh *et al.* [1932] pointed out that what was actually measured, for example in the case of iodine as oxidising agent, was the redox potential of the system $I_2 \rightleftharpoons 2I$. This criticism was substantiated by the experimental result that the E_0 depended upon the nature of the oxidising agent.

Ghosh *et al.* [1932] presented evidence that by the electrolytic reduction of cystine to cysteine at a mercury electrode a reversible system was set up, the potential of which was determined by the concentration of both the reduced and oxidised forms. The potentials were found to agree with the following equation:

$$E_0 = 0{\cdot}078 - \frac{RT}{F} p_H - \frac{RT}{F} \log \frac{\text{cysteine}}{\sqrt{\text{cystine}}}.$$

These observations of Ghosh *et al.* presented a curious dilemma. When a mixture of cysteine and cystine is prepared by electrolytic reduction, the potential is a function of both the SH and SS concentration, and the E_0 is $+ 0{\cdot}078$ volt. But when the same mixture of cysteine and cystine is prepared by solution of the crystalline substances, the potential is determined by the concentration of SH only, while the E_0 is $- 0{\cdot}001$ volt.

The obvious element of difference between the two mixtures is in the mode of formation of the cysteine. In the one case, cysteine is prepared *in situ* by the electrolytic reduction of a solution of cystine: in the other case, crystalline cysteine prepared by the reduction of cystine with tin and hydrochloric acid is dissolved in the mixture fluid.

Various possibilities presented themselves.

(1) The electrolytic reduction of cystine results in an active form of cysteine, which is in equilibrium with the disulphide.

(2) The electrolytic reduction of cystine involves formations other than that of cysteine, and these formations determine the reversible state.

(3) Electrolytic reduction of cystine may convert it into a form which is in equilibrium with the cysteine produced.

In this report, an experimental analysis is made of the reversible state of the cysteine-cystine system induced by the electrolytic reduction of cystine at a mercury electrode. In addition potential measurements on both glutathione and glycylcysteine are described.

APPARATUS.

The nitrogen gas was dispersed into very minute bubbles by means of a Jena filter, and passed through a tower of alkaline pyrogallol (Fig. 1). The

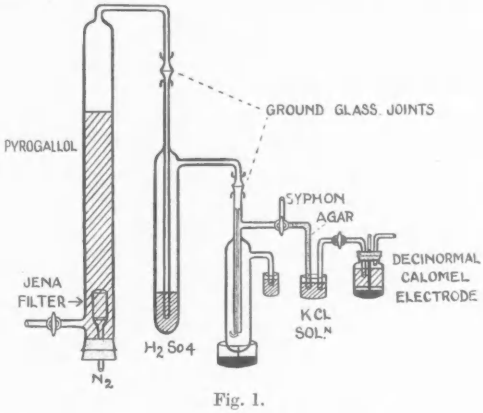

Fig. 1.

complete absorption of all traces of oxygen was attested by the failure of the issuing gas to develop any colour in an alkaline pyrogallol solution. Alkaline hydrosulphite solution was first employed for the removal of oxygen, but was discarded when examination of the issuing gas disclosed volatile sulphur compounds which affect the electrode considerably.

The cell was devised to avoid the use of either rubber-tubing or bungs. Air leakage, which is the most serious source of error in potential measurements of sulphydryl compounds, is eliminated by the ground glass joint connecting the cell with the rest of the apparatus. The inner portion of the cell consists of two tubes, one within the other. The inner tube is the gas inlet, and the outer tube the syphon for contact with the calomel electrode. Filling the syphon completely with fluid from the cell proved unsatisfactory in that the pressure in the cell forced liquid through the syphon into the beaker of KCl. This difficulty was overcome by filling the outer limb of the syphon with agar saturated with KCl. Contact of the agar with the fluid in the cell was made in the stopcock. The syphon served also for the withdrawal of samples of the contents of the cell for purposes of analysis. A burette was attached by means of rubber tubing to the limb of the syphon above the stopcock. Since the fluid within the syphon is not comparable in composition to the fluid in the cell proper, the first 3 cc. withdrawn were discarded. A platinum wire fused into the bottom of the vessel made electrical contact with the mercury electrode.

The apparatus is unique in its elimination of all but glass connections from the point of entry of the gas into the tower of pyrogallol to the point of exit from the cell. The cell permits of great ease and rapidity of manipulation.

For electrolysis a voltage of 200 volts was applied. The resistance of the circuit was sufficiently high to cut the amperage down to 5–20 milliamperes as shown by a milliammeter. By adjusting the position of the stopcock in the syphon, the amperage could be set to and maintained at any value in the above range. The cell with the mercury electrode served as the cathode while a beaker of saturated KCl with mercury at the bottom served as the anode.

METHODS.

Cystine, prepared from the hydrolysis of hair, was recrystallised three times by dissolving in dilute NaOH and precipitating with dilute acetic acid. Cysteine hydrochloride, prepared by the reduction of cystine with tin and hydrochloric acid, was purified by three recrystallisations from concentrated HCl. This procedure has been shown by Elvehjem [1930] to render the preparation metal-free.

The iodimetric method of Okuda [1925] was employed for the estimation of cysteine. Where the buffer power of the solutions estimated interfered with the conditions of p_H required for correct titration, new factors had to be determined.

The p_H values of the solutions were determined by the hydrogen and quinhydrone electrodes. In the more alkaline regions, only the hydrogen electrode could be relied upon.

The mercury used as electrode was purified by distillation *in vacuo* and cleaned successively with dilute nitric acid and distilled water.

A decinormal calomel half cell was used as the lead off electrode. The potential in the range of temperature between 17° and 20° was taken as + 0·338 volt with reference to the hydrogen electrode. The calomel electrode was calibrated against a quinhydrone-acetate half cell.

The instability of sulphydryl potentials makes it useless to measure with an accuracy greater than 0·5 millivolt. The Cambridge unipivot potentiometer box was found to be sufficiently sensitive for sulphydryl potentials. The accuracy is of the order of magnitude of 0·5 millivolt.

EXPERIMENTAL.

Cysteine potentials.

An attempt was made to repeat the respective measurements of Michaelis and Flexner, and of Ghosh *et al.*

An E_0 value of + 0·030 volt characterises the potentials of cysteine over a wide range of concentration and p_H (Table I). Michaelis and Flexner [1928] reported an E_0 of − 0·001 volt, but in some later experiments by Barron, Flexner and Michaelis [1929], in which the technique was slightly modified, an E_0 more positive by 20–30 millivolts was observed.

Table I.

Cysteine obtained by reduction with tin.

Molarity of cysteine	p_H	E_0 in volts
0·001	8·34	+0·029
0·010	7·17	+0·028
0·010	4·63	+0·031
0·010	6·11	+0·028
0·010	8·08	+0·035
0·010	7·35	+0·029
0·004	8·50	+0·030

Mean E_0: +0·030 volt.
Michaelis E_0: −0·001 volt.

There is fairly good agreement between the E_0 value in Table II and that of Ghosh *et al.* From the dependence of the potential on the ratio of reduced to oxidised component, it follows that a reversible system is set up as the result of the cathodic reduction of cystine at a mercury electrode.

Table II.

Mixtures obtained by cystine electrolysis.

Molarity of cysteine	Molarity of cystine	p_H	E_0 in volts
0·0023	0·0088	9·2	+0·077
0·0038	0·0001	8·5	+0·077
0·0038	0·0001	8·2	+0·077
0·0050	0·0010	8·5	+0·078
0·0038	0·0001	8·5	+0·080
0·0030	0·0005	8·5	+0·073

Mean E_0: +0·077.
Ghosh E_0: +0·079.

The question arose whether the reversibility depended upon some alteration of the mercury electrode rather than upon a true state of equilibrium between cysteine and cystine in the body of the liquid. The following experiments were performed to determine this point.

(1) 0·002 M solution of cystine in a borate buffer of p_H 8·50 was electrolysed for 1 hour at 5 milliamperes. The equilibrium value was − 0·372 volt. The final potential was usually reached in about 2 hours. The criterion for equilibrium was constancy of the potential to within 2 millivolts over a period of 1 hour. Samples of the fluid in the cell were removed, and from the concentration of cysteine determined by titration, the theoretical potential demanded by the Ghosh equation was calculated. The agreement was to a millivolt.

The cell was then disconnected at the glass joint and the contents removed. The fluid with fresh mercury was poured back into the cell, and the cell connected again with the rest of the apparatus. The equilibrium potential turned out to be $- 0{\cdot}324$ volt, which is precisely the potential demanded by the Dixon equation using the E_0 value of $+ 0{\cdot}030$ volt.

It is quite clear from this experiment, which was repeated many times, that by electrolysis an equilibrium system is set up at the mercury electrode, but that by renewal of the mercury electrode the condition of irreversibility is restored.

(2) 0·1 *M* cysteine hydrochloride was made up in a phosphate buffer of p_H 7·35. At equilibrium, a potential of $- 0{\cdot}281$ volt was reached. A current of 5 milliamperes was allowed to pass through the solution for 1 hour. After this treatment, the equilibrium potential amounted to $- 0{\cdot}305$ volt.

Since the solution contained the reduced form initially, the increase in negativity of the potential cannot be due to any increase in SH concentration. The only plausible explanation is that the mercury electrode itself has suffered some change. If this conclusion is correct, then if two mercury electrodes are used, one for electrolysis and the other for comparison, a serious discrepancy should be manifest in the equilibrium potentials recorded by the pair of electrodes.

(3) This experiment was carried out with the cell shown in Fig. 2. A discrepancy of 60 millivolts was observed in the equilibrium potentials for the two mercury electrodes. The one through which the current passed recorded an E_h of $- 0{\cdot}398$ volt which agreed with the Ghosh equation, whereas the control recorded an E_h of $- 0{\cdot}338$ volt which agreed with the Dixon equation.

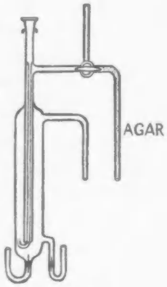

Fig. 2.

The nature of this alteration at the surface of the mercury electrode during electrolysis was investigated. The following experiments of a rough qualitative nature were carried out.

(1) Mercury was placed in a series of test-tubes containing solutions of cysteine, cystine and reduced and oxidised glutathione in phosphate buffer. The tubes were shaken vigorously for 3 minutes. The supernatant liquid was decanted and tested with H_2S. In all cases, a precipitate of mercuric sulphide resulted. If instead of shaking with air, nitrogen gas from a tank was bubbled through, hardly any precipitate formed. Purified nitrogen bubbled through the mercury for 30 minutes did not effect any observable solution of mercury.

It is quite clear that complex formation of mercury with either SH or SS compounds takes place rapidly in the presence of oxygen. In the complete absence of oxygen, no complex formation ensues from shaking mercury with sulphydryl solutions.

Barron, Flexner and Michaelis [1929] have shown that complex formation in the complete absence of oxygen can take place if a galvanic cell arrangement is set up. Cysteine in contact with mercury forms one half cell and a calomel electrode the other. The potential difference between the cysteine solution and the calomel directs a flow of current from the calomel to the cysteine half cell. If current is allowed to pass for several hours, a distinct coloration of the solution with H_2S is obtained, even though purified nitrogen has removed all traces of oxygen from the cysteine half cell.

This experiment was repeated with cysteine and reduced glutathione. In both cases, the formation of a mercury complex was confirmed. It is noteworthy that in electrolysis the flow of current is opposite to the flow in the galvanic cell. The observation of Ghosh *et al.* that after electrolysis of cystine H_2S does not form a precipitate was confirmed. But their conclusion from this negative test that no complex formation took place was hardly justified. If mercury goes into solution in a galvanic cell, it may not be unreasonable to expect that with the current flowing in the opposite direction as in electrolysis, the cysteine will go into the surface of the mercury, forming some complex which is confined to the mercury surface. If that were the case, renewal of the surface should eliminate the complex. The experiments described above confirm this prediction.

It may be concluded from these observations that during electrolysis some complex of mercury and cysteine is formed. This complex determines the reversibility of the system. It is conceivable either that this complex is in equilibrium with cysteine and cystine, or that the complex acts as a catalyst in promoting a state of equilibrium between the cysteine and the cystine. By removing the mercury used for electrolysis, the complex is likewise removed and the new mercury electrode attains a potential which depends only upon the concentration of SH.

Colorimetric measurements.

The other experiments on the cysteine potential are concerned with the agreement between the electrode potentials and the potentials determined by the reduction of reversible indicators. It is of great importance in the proper analysis of the cysteine potential to know whether cysteine can reduce dyestuffs to the degree demanded by the formula.

The experiments were carried out in Thunberg tubes. To insure the complete removal of all traces of oxygen, the tubes were three times exhausted and filled with purified nitrogen.

The data in Tables III and IV show that the reducing power as measured by indicators is less than that predicted by the electrode equation. However, the increase in reducing power with increase in concentration seems closely parallel with the electrode phenomena.

Table III.

Cysteine HCl; p_H 7·0; 30°

Indicator	r_H	0·001 *M*	0·010 *M*	0·100 *M*	0·500 *M*
O-Cresolindophenol	20·5	Reduced	Reduced	Reduced	Reduced
Cresyl blue	15·1	,,	,,	,,	,,
Methylene blue	14·4	,,	,,	,,	,,
Janus green A	—	,,	,,	,,	,,
Indigo carmine	9·9	Partial reduction	,,	,,	,,
Nile blue	9·2	No reduction	Partial reduction	,,	,,
Cresyl violet	8·3	,,	,,	,,	,,
Phenosafranine	5·7	,,	No reduction	No reduction	,,
Janus green B	5·2	,,	,,	,,	,,
Neutral red	2·3	,,	,,	,,	No reduction

Table IV.

Cysteine HCl; p_H 7·0; 30°

Molarity	Calculated r_H	Observed r_H
0·001	7·0	9·9
0·010	5·0	8·5
0·100	3·0	6·6
0·500	1·6	3·5

$$r_H = \frac{E_h + 0{\cdot}060\, p_H}{0{\cdot}030}$$

$$E_h = 0{\cdot}030 - 0{\cdot}060\, p_H - 0{\cdot}060 \log \mathrm{CySH}.$$

GLUTATHIONE POTENTIALS.

Dixon and Quastel [1923] studied the properties of impure glutathione.

In Table V are recorded the potentials exerted by solutions of pure crystalline glutathione [Hopkins, 1929] of various concentrations and of various degrees of acidity and alkalinity at a mercury electrode. The E_0 value is 30 millivolts more positive than that of cysteine. The potentials agree closely with the following equation:

Table V.

Glutathione

Molarity	E_h observed in volts	E_h calculated in volts	p_H	E_0 in volts
0·001	−0·0345	−0·0320	4·63	0·0596
0·001	−0·1220	−0·1228	6·22	0·0630
0·001	−0·1685	−0·1675	6·90	0·0628
0·001	−0·1740	−0·1742	7·09	0·0624
0·001	−0·2162	−0·2168	7·96	0·0620
0·001	−0·1725	−0·1711	7·00	0·0636
0·010	−0·2328	−0·2326	7·09	0·0624
0·005	−0·2123	−0·2110	7·09	0·0635
0·005	−0·1645	−0·1644	7·09	0·0623
0·020	−0·2510	−0·2522	7·09	0·0610

Mean $E_h = +0·0622$ volt.

$$E_h = 0·0622 - \frac{RT}{F} p_H - \frac{RT}{F} \log GSH.$$

The following experiment was performed to prove the inactivity of the oxidised form at the mercury electrode. The cell was filled with 0·001 *M* reduced glutathione in phosphate buffer of p_H 7·09. The equilibrium potential was − 0·174 volt. The cell was disconnected and 62 mg. of oxidised glutathione were added to the contents through the gas inlet tube, making the solution 0·001 *M* with respect to the oxidised form. The equilibrium potential remained at its original value of − 0·174 volt.

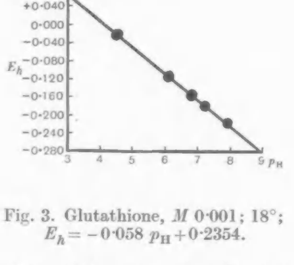

Fig. 3. Glutathione, *M* 0·001; 18°; $E_h = -0·058\ p_H + 0·2354$.

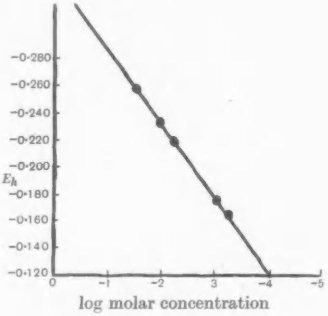

Fig. 4. Glutathione, p_H 7·09; 18°; $E_h = -0·3470 - 0·058 \log GSH$.

Fig. 4 shows graphically the variation in the potential with changes in the molar concentration of glutathione at p_H 7·09. Fig. 3 shows the variation in the potential with changes in the p_H of 0·001 *M* glutathione. The linear relations observed agree closely with the electrode equation.

Colorimetric measurements.

A comparison of the electromotive activity and the reducing power as measured by indicators was also made. The technique was the same as that described for the study with cysteine.

Table VI.

Glutathione; p_H 7·0; 30°

Indicator	E_0 at p_H 7·0	r_H	0·01 M	0·10 M
O-Cresolindophenol	+0·195	20·5	Reduced	Reduced
Cresyl blue	+0·032	15·1	,,	,,
Methylene blue	+0·011	14·4	,,	,,
Janus green A	—	—	,,	,,
Indigo carmine	−0·125	9·9	,,	,,
Nile blue	−0·142	9·2	No reduction	,,
Cresyl violet	−0·167	8·3	,,	,,
Phenosafranine	−0·242	5·7	,,	No reduction
Janus green B	−0·258	5·2	,,	,,
Neutral red	−0·340	2·3	,,	,,

Table VII.

Glutathione; p_H 7·0; 30°

Molarity	Calculated r_H	Observed r_H
0·0001	10·0	—
0·0010	8·0	—
0·0100	6·0	9·3
0·1000	4·0	6·6

$$r_H = \frac{E_h + 0{\cdot}060\, p_H}{0{\cdot}030}.$$

$$E_h = 0{\cdot}0622 - 0{\cdot}420 - 0{\cdot}060 \log \text{GSH}.$$

It is clear from Tables VI and VII that there is some discrepancy between the potentiometric and colorimetric data. Several indicators which should be completely reduced by 0·01 M glutathione, for example Nile blue, are not even partially reduced. However there is an increase in reducing power with increase in the molarity of glutathione as demanded by the electrode formula. Qualitatively colorimetric data compare favourably with potentiometric data, but quantitatively the data are hardly concordant.

GLYCYLCYSTEINE POTENTIALS.

The difference in E_0 values for cysteine and glutathione raised the interesting question of the effect of the number of amide linkages and of the nature of the linkage upon the potentials of cysteine peptides. Cysteinylglycine, which is intermediary between cysteine and glutathione, has never been successfully prepared. Glycylcysteine has been prepared in fairly pure state by Pirie [1931]. This peptide involves an amide linkage between the amino-group of cysteine and the carboxyl group of glycine, whereas in glutathione the amino-group of glycine is coupled with the carboxyl group of cysteine.

In Table VIII are recorded the potential measurements of solutions of glycylcysteine. Surprising indeed is the fact that the E_0 value is even more negative than that of cysteine. Apparently the number of peptide linkages is not a determining factor of the reducing power of cystine derivatives. What is more likely to be the controlling factor is the nature of the peptide linkage.

Table VIII.

Glycylcysteine.

Molarity	E_h observed in volts	E_h calculated in volts	p_H	E_0 in volts
0·0013	−0·2170	−0·2168	7·09	0·0250
0·0100	−0·2670	−0·2673	7·09	0·0255
0·0500	−0·3085	−0·3088	7·09	0·0255
0·0100	−0·1253	−0·1251	4·63	0·0250
0·0100	−0·3950	−0·3948	9·28	0·0250
0·0100	−0·3211	−0·3211	8·00	0·0252
0·1000	−0·3263	−0·3264	7·09	0·0253

Mean $E_0 = +0{\cdot}0252$ volt.

The typical irreversibility of sulphydryl compounds is also shown by glycylcysteine. In Figs. 5 and 6 are shown the relationship of the variables in the electrode formula

$$E_h = 0{\cdot}025 - \frac{\mathrm{RT}}{\mathrm{F}}\, p_{\mathrm{H}} - \frac{\mathrm{RT}}{\mathrm{F}} \log \mathrm{RSH}.$$

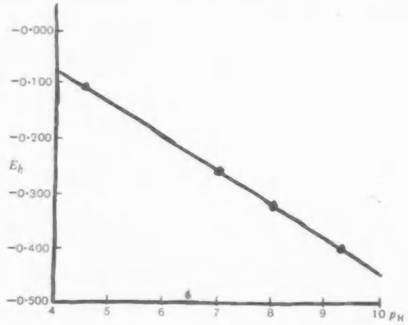

Fig. 5. Glycylcysteine, 0·01 *M*; 18°; $E_h = 0{\cdot}1407 - 0{\cdot}058\ p_{\mathrm{H}}$.

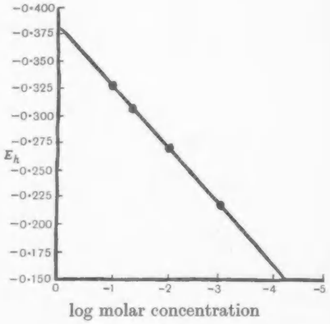

Fig. 6. Glycylcysteine, p_{H} 7·09; 18°; $E_h = -0{\cdot}3870 - 0{\cdot}058 \log \mathrm{RSH}$.

DISCUSSION.

Dixon and Quastel [1923] in an attempt to explain the peculiarities of the cysteine potential, assumed that the oxidation of cysteine to cystine took place in two distinct steps.

$$(1)\ \mathrm{RS}^- \rightleftharpoons \mathrm{RS}^* + E.$$
$$(2)\ 2\mathrm{RS}^* \longrightarrow \mathrm{RSSR}.$$

The first step, involving the oxidation of the anion of cysteine to the free radical, is reversible. The second step, involving the condensation of the free radical to cystine, is irreversible. Thus it follows from the equation that for a constant p_{H} the potential is determined solely by the concentrations of cysteine and its primary oxidation product, *viz.* RS*. To account for the experimental result that cysteine alone determined the potential, Dixon and Quastel [1923] further assumed that the concentration of the primary oxidation product is both small and constant. The solution will then always be saturated with primary oxidation product.

Michaelis has objected to this theory on the following grounds. (1) If the irreversibility of the second reaction is absolute, then at equilibrium RS* is zero and the potential should be *minus* infinity. (2) If the irreversibility is relative, changes in the concentration of cystine should affect the potential. The experimental results are not in accordance with either of these predictions from the theory.

Dixon [1927] later offered another theory which is based on his observations that the potential of a cysteine solution is approximately 200 millivolts more negative against mercury than against an electrode of pure gold. It is well known that gold and mercury differ strikingly in their capacity for hydrogen over-voltage, and Dixon attempted to relate the differences in the cysteine potentials at these metals to the differences in this property.

Michaelis objected to this theory also on the following grounds. (1) The potentials reached by all electrodes except gold are identical provided all traces of oxygen are removed. (2) If the potential depends on hydrogen over-voltage, then substituting hydrogen gas for nitrogen should lower the potential to a more negative value. In point of fact, the potential at mercury is exactly the same whether a current of nitrogen or hydrogen be passed through the solution. (3) If the potential is determined by equilibrium between the rate at which cystine charges the electrode with hydrogen atoms and the rate at which hydrogen escapes from the electrode as gas, it should be possible to vary the potential by addition of a catalyst increasing the reduction velocity of cysteine. Harrison and Quastel [1928] have shown, however, that additions of small amounts of iron and copper do not appreciably alter the potential of a metal-free cysteine solution.

Michaelis [1929] has offered his own theory to explain the anomalies of the cysteine potential. He postulates that the potentials of cysteine solutions in the presence of an electrode are due to metal complexes of cysteine and not to cysteine alone. Barron, Flexner and Michaelis [1929] have amassed a great deal of evidence for the formation of complexes of cysteine with mercury, platinum and other metals and consider the evidence as sufficient proof of the theory.

The theory, however, has many serious drawbacks. (1) It seems very improbable that if the potential of cysteine solutions involved metal complexes, the same potential should be reached whether mercury, platinum or gold be used as the electrode. The chances that complexes of such different metals should be equivalent electromotively are very slight indeed. (2) The aberrations of the cysteine potential at a p_{H} of 9 and higher have been explained as due to the dissociations of cysteine. Michaelis and Flexner [1928] have observed this irregular behaviour of the potential in the more alkaline regions, regardless of the type of electrode used. It is certainly difficult to believe that complexes of cysteine with a variety of electrode metals should all possess approximately the same dissociation constants. The variation of the potential with the p_{H} would be expected to depend upon the nature of the electrode used, which it does not. (3) The evidence is hardly conclusive that complex formation takes place under the conditions of the experiments in which potential measurements are made. That complexes are formed as a result of the passage of a current or as the result of shaking cysteine and mercury in the presence of oxygen does not necessarily imply complex formation in the absence of oxygen and the absence of any current. (4) If the potential of cysteine solutions in the presence of mercury depends on the ratio $\frac{[\mathrm{RSH}]}{[\mathrm{HgS_2R_2}]}$, the addition of a saturated solution of the mercury complex should change the potential enormously. In point of fact, Barron, Flexner and Michaelis [1929] failed to observe a change of more than a few millivolts. (5) All known oxidation-reduction systems which are reversible have a span of potentials from the 95 % reduced to the 95 % oxidised state of approximately 70 millivolts. The cysteine potentials, however, can be made to spread over 300–400 millivolts by progressive increases in concentration. It is quite clear that the ordinary type of reversible system cannot explain the cysteine potential. If as Barron, Flexner and Michaelis postulate, the electrode reaction is as follows

$$2RSH + Hg \rightleftharpoons (RS)_2Hg + 2H$$

it is difficult to see why this reversible system should have a span of potentials many times that of the methylene blue system, for example. In going from a state of 95 to 100 % oxidation, the potentials of the methylene blue system vary by a matter of 10 millivolts. Since the concentration of $(RS)_2Hg$ must be extremely small compared to the concentration of cysteine, the Michaelis theory virtually attributes a span of 300–400 millivolts to the change from about 99·9 to 100 %. This of course makes the theory very difficult to accept.

In the usual type of reversible system, two processes are at work at the electrode; the reductant charging the electrode with electrons, and the oxidant discharging the electrode. That is to say there are two mutually compensating processes at the electrode. In practice, it is almost impossible to get either pure oxidant or pure reductant—which means that there are limits to either the positivity or negativity which the system can attain.

Sulphydryl systems lack that simple condition. In terms of electrons, the inactivity of cystine at the electrode means that it cannot discharge the electrons released by cysteine. From electrolysis experiments it is known that only when the electron pressure is comparatively enormous does cystine accept the electrons at the mercury surface. Therefore cysteine builds up an electron pressure, so to speak, which is not in equilibrium with cystine. However, there must be some compensating process, for otherwise the potential would be infinitely negative. Also this compensating process must be constant whatever the concentration of cysteine, for otherwise it would be impossible to get increasingly negative potentials with increases in the concentration of cysteine. What remains to be ascertained is the nature of this compensating process at the electrode. It is noteworthy that the theory of Dixon [1927] is the only one yet proposed which provides a compensation mechanism for the cysteine potential. The theory of Michaelis [1929] completely overlooks this special aspect of the cysteine potential.

The colorimetric measurements with glutathione suggest that the potentials of animal tissues may very well be determined by this sulphydryl compound. A 0·01 *M* solution of glutathione at p_H 7·0 has an r_H of 9·3 which is approximately the anaerobic r_H of tissues.

SUMMARY.

1. The reversibility claimed by Ghosh *et al.* for the cysteine-cystine system prepared by electrolytic reduction has been subjected to experimental analysis. It has been shown:

(*a*) That the mercury electrode employed for the electrolytic reduction undergoes some alteration.

(*b*) That with renewal of the mercury electrode, the potentials do not conform with the equation for the reversible state.

(*c*) That after passage of current through a mercury electrode in contact with a solution of cysteine, the equilibrium potentials of the mercury electrode are shifted to more negative values.

(*d*) That in a solution containing a mixture of cysteine and cystine prepared by electrolytic reduction, the potential at the mercury electrode through which the current has passed agrees with the equation of Ghosh *et al.*, whereas the control mercury electrode agrees with the Dixon and Quastel equation.

2. The colorimetric and potentiometric values for the cysteine potential are not in agreement. Potentiometric values are more negative by approximately 90 millivolts or 3 r_H units. However, the increase in the reducing power of cysteine with increase in the molar concentration, parallels the electrode phenomena closely.

3. Potential measurements on both glutathione and glycylcysteine are recorded. These two sulphydryl compounds exert negative potentials which depend only upon the concentration of reduced component. The E_0 of glutathione is + 0·062, and of glycylcysteine + 0·025.

4. The colorimetric and potentiometric values for the glutathione potential are not in agreement. The potentiometric values are more negative by approximatively 60 millivolts or 2 r_H units. Qualitatively, however, colorimetric and potentiometric data are comparable.

5. The various theories proposed to explain the anomalies of sulphydryl potentials have been reviewed. A suggestion has been made as to the lines of approach which a complete analysis must follow.

I am grateful to Sir Frederick Hopkins for his encouragement and to Dr Malcolm Dixon for his stimulating suggestions and constant interest in the course of the investigation.

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XC. THE EFFECT ON THE BLOOD-SUGAR AND BLOOD-PHOSPHATE OF INGESTED CREATINE.

By REGINALD STEPHEN STACEY.

From the Medical Unit, St Thomas's Hospital.

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THE variations in the level of the inorganic phosphate of the blood after the ingestion of glucose and the different responses obtained in the normal and in the diabetic organism are now well established [Harrop and Benedict, 1924 and others]. The reverse of this, namely, the effect of ingested phosphate on the blood-sugar has been investigated by Elias and Weiss [1922], Magenta and Biasotti [1924] and by Friedländer and Rosenthal [1926], but the results are not in sufficiently good agreement for any definite conclusion to be drawn. The fact that creatine is now known to be associated with phosphorus in the metabolic processes of muscle [Eggleton and Eggleton, 1928; Fiske and Subbarow, 1929] suggested the possibility that some light might be thrown on the metabolism of creatine and carbohydrate by comparing the action of administered creatine on the blood-sugar and blood-phosphate in normal and diabetic patients.

Some experiments on this subject with normal animals have already been reported. Hill and Mattison [1929] have shown that in dogs, doses of creatine as small as 0·025 g. per kg. given by mouth produce a fall in the blood-sugar provided its initial value is not very low, whilst Brown and Imrie [1931] obtained a fall in the phosphate excretion in the urine after giving creatine to anaesthetised cats. These latter results were not, however, confirmed by Fasold and Loeschke [1932], who examined the effect of creatine on the phosphate excretion of adults and children. They came to the conclusion that in neither was there any change in phosphate excretion and that the changes observed by Brown and Imrie were probably due to the large volume of water given with the creatine.

Method.

The experiments described here were carried out between the hours of 9 a.m. and 1 p.m. on patients who had fasted since the preceding evening. In the case of the diabetic patients no insulin had been given for 15 hours. A sample of blood was taken at the beginning of the experiment from the arm and oxalated to 2 mg. per cc. The patient then drank 2–5 g. creatine in 100 cc. water, and further samples of blood were taken up to 3–4 hours after the beginning of the experiment.

Glucose was determined on the whole blood by MacLean's method [1919]. The plasma was then separated, and the inorganic phosphate and total creatinine (creatine *plus* preformed creatinine) were determined in it by the methods of Fiske and Subbarow [1925] and of Folin and Wu [1919] respectively.

Results.

The results of the blood analyses are collected in Table I, in which the inorganic phosphate is expressed as mg. of phosphorus per 100 cc. The preformed creatinine was determined in several experiments and was found to be constant.

Table I.

Normals

Exp.	Time after creatine min.	Dose g.	Blood-sugar mg./100 cc.	Plasma-inorganic P mg./100 cc.	Plasma-total creatinine mg./100 cc.
1	0	2·0	90	4·00	2·15
	60		104	3·52	4·85
	140		104	3·69	3·84
	185		137	4·06	3·65
2	0	3·0	102	3·92	2·31
	65		125	3·84	4·16
	130		117	4·14	3·68
	200		106	3·81	3·14
4	0	3·0	111	3·53	2·23
	60		92	3·33	6·50
	140		104	3·43	4·13
5	0	3·0	106	4·38	2·27
	60		106	4·13	5·20
	140		97	4·56	4·13
6	0	4·0	129	4·54	1·90
	60		125	3·92	4·30
	120		122	3·88	8·05
	180		113	3·62	6·10
7	0	4·0	115	4·22	2·30
	100		120	3·83	6·30
	155		109	4·03	6·50
	225		117	3·98	4·75
8	0	4·0	106	3·90	3·10
	60		109	3·24	6·90
	115		92	3·24	6·10
	200		102	3·28	6·75
9	0	5·0	102	3·79	2·45
	60		100	3·76	8·00
	120		77	3·57	8·35
	180		69	3·62	7·00

Diabetics

Exp.	Time after creatine min.	Dose g.	Blood-sugar mg./100 cc.	Plasma-inorganic P mg./100 cc.	Plasma-total creatinine mg./100 cc.
11	0	3·0	216	3·92	2·20
	60		291	3·95	4·10
	120		286	4·02	4·16
	180		299	4·00	3·60
12	0	2·0	171	3·29	4·60
	60		181	3·21	4·80
	145		156	—	—
13	0	3·0	254	3·28	1·86
	60		227	3·08	6·80
	120		179	3·10	4·78
	160		177	3·10	4·23
14	0	3·0	450	3·82	2·23
	75		440	2·80	5·20
	165		480	2·75	5·78
15	0	4·0	450	3·70	2·15
	55		441	3·70	6·10
	175		457	3·54	6·25
16	0	4·0	180	3·50	2·00
	60		180	3·10	7·75
	120		162	2·90	6·70
	180		138	3·11	5·50
17	0	5·0	296	3·82	2·85
	60		291	3·70	5·30
	120		286	3·55	9·25
	180		274	3·38	—
18	0	5·0	378	3·78	3·05
	60		390	3·44	8·30
	120		363	3·28	8·50
	175		383	3·36	8·00

	Normals mg./100 cc.	Diabetics mg./100 cc.
Average change in blood-sugar during 1st hr.	+2	+6
2nd hr.	−6	−9
3rd hr.	+4	−1
Average change in plasma-inorganic phosphate during 1st hr.	−0·34	−0·29

An approximate estimate of the rise in the plasma-creatine (expressed as creatinine) can be obtained by subtracting 1·5 from the figures in the total creatinine column, *e.g.* in Exp. 1, the rise was from 0·65 to 3·35 and in Exp. 9 from 0·95 to 6·85.

Blood-sugar. It will be seen that the blood-sugar varied very considerably during the course of an experiment, the variations being larger in the case of the diabetics than in the case of the normals. There is no evidence, however, of a tendency for it to rise or fall after creatine in either type of patient, and the averages for both show practically no change.

Plasma-inorganic phosphate. With the exception of one experiment there is a fall in the inorganic phosphate in every case although in three experiments the drop is less than 5 %. It can therefore be said that in thirteen experiments out of fifteen a definite, though in no case very large, drop was obtained (Exp. 12 is not considered, as the drop during the second hour is not known). Further, in five out of six experiments in which the plasma-creatine reached its maximum at the end of the first hour, the phosphate started to return to normal during the second, and out of the nine experiments in which the creatine continued to rise during the second hour, in eight of them the phosphate continued to drop.

While there is a marked tendency for the phosphate and creatine to vary together, a rise in the plasma-creatine being accompanied by a fall in the phosphate, the fall in the phosphate is not proportional to the rise in the creatine, as is seen by comparing Exps. 9 and 17, where there is a small phosphate drop, with Exps. 1 and 4, where it is large.

Comparing the normals with the diabetics no definite differences are to be seen.

Although the changes obtained are small it is considered that the good agreement of the results eliminates the possibility that they are chance effects, and the conclusion is drawn that a small drop in the plasma-inorganic phosphate follows the ingestion of creatine, but it is by no means constant in amount and is probably influenced by some unknown factor.

SUMMARY.

1. The blood-sugar and plasma-inorganic phosphate have been estimated before and after the ingestion of creatine by normal and diabetic patients.

2. After creatine the blood-sugar shows no decided change in either direction.

3. The inorganic phosphate tends to fall as the creatine content of the plasma rises.

4. Normals and diabetics react essentially in the same way.

5. The phosphate fall does not vary quantitatively with the rise in the plasma-creatine.

I am indebted to Prof. de Wesselow for facilities with regard to patients and for his interest and advice.

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XCI. THE ANTISCORBUTIC POTENCY OF APPLES. VI.

By THOMAS WALLACE and SYLVESTER SOLOMON ZILVA[1].

From the Agricultural and Horticultural Research Station, Long Ashton, Bristol and the Division of Nutrition, Lister Institute, London.

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Preliminary observations made in connection with this inquiry demonstrated that, whilst Bramley's Seedling apples were antiscorbutically more active than King Edward apples, both varieties being typical late culinary sorts, their nitrogen content was definitely lower [Bracewell, Hoyle and Zilva, 1930, 1, 2]. In consequence of this observation we attempted [Bracewell, Wallace and Zilva, 1931] to ascertain whether by changing the nitrogen contents of fruits of these varieties their antiscorbutic activities could at the same time be influenced. It was found that in the case of the King Edward variety the vitamin C content was indeed higher in the apples in which the nitrogen content was lowered by cultural treatment, *i.e.* by substituting "grass" for arable culture. The difference in the antiscorbutic potency between the high and low nitrogen apples of the Bramley's Seedling variety was, however, very much less marked and its magnitude fell within the limits of the experimental error.

These results although not sufficiently clear-cut to be conclusive were suggestive enough to make it advisable to pursue the subject further. The experiments have therefore been repeated and extended during the last 3 years. In these further tests the nitrogen contents of the two varieties previously used have been varied by two methods; by substituting "grass" for arable culture and by the operation of bark-ringing vigorous trees. In these two cases the "grass" and bark-ringing practices lower the nitrogen contents of the fruits and at the same time usually increase the percentages of dry matter and total sugars [Wallace, 1930; Wallace, Charley and Jones, 1930], bark-ringing being generally more effective in increasing the latter two constituents. The results we have obtained are in our opinion definite enough to support the view that in the case of apple varieties an inverse relationship exists between the nitrogen content and the vitamin C activity.

Experimental.

A detailed description of the procedure followed in the vitamin C tests and their assessment has been given already in a previous communication of this series [Bracewell, Hoyle and Zilva, 1930, 1]. In all tests the fruits immediately after picking were placed in a low temperature store and kept at 1°. The humidity of the chamber was maintained at about 95 %. Samples for testing purposes were drawn from the store at weekly intervals. The chemical determinations in all cases were made at the time of placing the samples in store. The material for analyses, in every case, was obtained from 25 fruits per sample by removing a thin layer of peel and cutting thin slices across the fruits at right angles to the core, slices for nitrogen determinations always being taken at the equatorial regions and those for sugars, acid and ash from either side of the nitrogen regions in this order. The following are the cultural details relating to the trees from which the samples were obtained.

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[1] Member of the Scientific Staff, Medical Research Council, working with grants for assistance and expenses.

1930. Bramley's Seedling.

Arable, high nitrogen sample. The fruits were grown at Ledbury, Herefordshire. The soil is a heavy loam containing large proportions of the fine sand and silt fractions and is derived from marl deposits of the Old Red Sandstone formation. It contains a small supply of calcium carbonate. The trees are well grown half standards, from 25 to 30 years old, and since planting they have always been under high cultural conditions. Manuring until a year or two previous to 1930 had been very heavy, especially large dressings of nitrogen and phosphorus being applied, and the trees reflected the treatment. During the season of taking the samples, growth was vigorous and all trees carried a heavy crop of medium sized, green fruits. The fruits selected for the experiment were typical of the crop, being only of medium size, green with an occasional dull flush, juicy and of sharp flavour. Picking was carried out on October 15th. During the period of the test the fruits remained in a fresh condition and no storage breakdown occurred.

Grass, low nitrogen sample. This sample was obtained from an orchard at Marston Magna, Somerset. The soil is a heavy loam with a clay subsoil and is mainly derived from Lower Lias clay. There is a surface wash present consisting of mixed material of Middle Lias and Lower Lias origin, the sand of the former serving to lighten the surface layer. The soil is slightly calcareous. The trees are of the bush form and had been planted in 1912. Until 1917, the plantation was maintained under arable conditions but from then until the year of sampling the trees had been under grass culture with occasional stocking and had degenerated into typical low nitrogen specimens. No manures had been applied to the area while the trees were under grass.

In 1930, the trees were of poor vigour, there being practically no shoot growth, and the foliage was of yellowish-green colour. The crop was heavy and the fruits medium in size with pale ground colour and generally a brilliant red flush, typical of low nitrogen conditions. The flesh was white and firm and the flavour sweet. Picking was carried out on October 14th. The fruits remained in good condition during the test, and no breakdown was recorded.

Not ringed, high nitrogen sample. These fruits were grown near Wisbech under conditions typical of the district. The soil is a typical heavy silt-clay with a characteristic high water table. The trees are of the bush type and were approximately 20 years old at sampling. They had been grown under high cultural conditions until a season or two before, when cultivation was lowered to reduce the vegetative growth. Manuring had not been heavy in view of the natural vigour of the trees on this type of soil. In 1930 the trees were growing strongly and the foliage was dark green in colour indicating high nitrogen conditions. The trees selected for the test carried a medium crop, the size of the fruits being mostly medium, the colour dark green and the flesh juicy and acid to taste. Picking was done on October 17th. During the test all fruits remained fresh and sound in store.

Ringed, low nitrogen samples. This sample was gathered from trees in the same orchard as the previous "not ringed" sample and all conditions for growth *etc.* were comparable in the two cases with the exception of the "ringing" treatment which had been performed during May 1930. The effect of the ringing was typical and very marked; shoot growth was greatly restricted and the leaves were pale green, and during the latter part of the season showed high yellow, red and purplish tints. The fruits had the appearance of low nitrogen specimens, possessing a very pale ground colour with bright red flush, while the flesh was white, hard and notably sweet for the variety. The crop was again medium although slightly less than the "not ringed." Picking was carried out on October 17th. Towards the end of the test, many fruits showed symptoms of "Flesh Breakdown" and at the conclusion of the experiment an examination was made with the following results; 30 fruits cut showed 19 with "Flesh Breakdown" to a medium extent and 1 with "Bitter Pit."

1931. Bramley's Seedling.

Not ringed, high nitrogen sample. This sample was secured from the same plantation at Faversham, Kent, as the 1929 Bramley, high nitrogen fruits [see Bracewell, Wallace and Zilva, 1931].

In 1931, the cultivation differed from that of 1929, the cultural system having been changed in 1930 from "arable" to "grass" with a view to lowering the highly nitrogenous growth of the trees. Nevertheless, in 1931, the trees were still growing vigorously and bearing excellent green foliage. The crop was heavy, the fruits being of medium size, green in colour with an occasional dull brownish-red flush. The flesh was juicy and fairly acid. Picking was carried out on September 19th. The fruits appeared fresh throughout the test and at the conclusion a storage examination showed the following results; 50 cut fruits gave 4 very slight "Core Flush" and 1 very slight "Flesh Breakdown."

Ringed, low nitrogen sample. These fruits were from trees comparable in every way with those of the previous sample with the exception that the trees in this case had been "ringed" in May, 1931. The effect of the ringing treatment was again very pronounced, both on the growth characters of the trees and the quality of the crop. The effects of the ringing were as described in the 1930 experiment. The crop was heavy, fruit size was slightly larger than in the "not ringed" sample and the fruits were highly coloured, white fleshed and very sweet to taste. Some storage breakdown and a certain amount of slight "Superficial Scald" developed during the later stages of the experiment, an examination at the conclusion yielding the following results; 50 fruits cut showed 32 slight, 1 medium and 2 severe cases of "Flesh Breakdown."

It should be noted that the flavour of "breakdown" specimens was fairly fresh and suggested plentiful supplies of acid and sugar.

1932. King Edward VII.

Not ringed, high nitrogen sample. The fruits of this sample were taken from the same group of trees at Long Ashton Research Station as those used for the arable, high nitrogen series in the 1929 experiments. The cultural and other treatments practised on the plot previous to 1929 had been maintained until 1931 without any change, and the trees had continued to grow vigorously.

The 1932 crop was medium and the fruits were of medium size and bright green in colour with an occasional dull brownish-red flush. The flesh was fairly juicy and the flavour medium acid and typical of the variety. Picking was done on October 14th. No storage breakdown occurred during the period of the test.

Ringed, low nitrogen sample. These fruits were from the same group of trees as those of the previous sample, the only difference being the ringing treatment. In order to obtain a large effect, the trees were "ringed" in two consecutive

Table I.

Season	Plantation	Variety	Special treatment	As % fresh weight						Ash in dry matter	Ash constituents as % ash				Ratio total sugars/ nitrogen
				Dry matter	N	Acidity as malic acid	Reducing sugars	Sucrose	Total sugars R+S		CaO	MgO	K_2O	P_2O_5	
1929	Faversham, Kent	Bramley's Seedling	Arable (high N)	11·9	0·0388	1·135	5·84	2·27	8·11	1·8	2·4	2·6	53·7	9·6	209
	Faversham, Kent	Bramley's Seedling	Grass (low N)	11·5	0·0169	0·838	6·69	1·97	8·66	1·6	3·8	2·5	50·9	12·0	512
1929	Long Ashton, Somerset	King Edward VII	Arable (high N)	13·5	0·0389	0·968	6·80	2·37	9·17	1·6	2·4	2·1	53·0	9·0	236
	Bratton, Wiltshire	King Edward VII	Grass (low N)	16·8	0·0307	0·750	10·28	1·97	12·25	1·1	3·1	3·1	50·4	10·7	399
1930	Ledbury, Herefordshire	Bramley's Seedling	Arable (high N)	11·4	0·058	0·79	7·26	1·28	8·54	1·29	3·02	3·38	53·5	12·8	147
	Marston Magna, Somerset	Bramley's Seedling	Grass (low N)	12·5	0·036	0·73	7·57	1·86	9·43	1·29	—	—	47·1	16·2	262
1930	Wisbech, Cambs.	Bramley's Seedling	Not ringed (high N)	10·9	0·059 (Dupl. sample)	0·99	5·92	1·79	7·71	1·98	—	—	55·3	9·62	131
	Wisbech, Cambs.	Bramley's Seedling	Ringed (low N)	13·8	0·044	1·00	6·75	3·38	10·13	1·47	—	—	56·5	7·45	230
1931	Faversham, Kent	Bramley's Seedling	Not ringed (high N)	12·0	0·039	1·18	6·59	2·28	8·87	1·45	2·50	—	50·2	14·1	227
	Faversham, Kent	Bramley's Seedling	Ringed (low N)	14·7	0·031	1·14	7·00	3·43	10·43	1·06	2·44	—	54·6	10·0	336
1932	Long Ashton, Somerset	King Edward VII	Not ringed (high N)	12·0	0·031	0·93	6·29	2·26	8·55	1·52	2·48	2·39	53·9	10·15	276
	Long Ashton, Somerset	King Edward VII	Ringed (low N)	13·1	0·025	0·77	7·83	2·76	10·59	1·09	2·77	2·72	52·0	11·23	424

years, in May 1931 and 1932. The treatment produced very marked effects both on growth characters and fruit quality during 1932. Shoot growth was entirely checked, the foliage was pale and became highly tinted with yellow, red and purplish shades. The crop was slightly heavier than on the "not ringed" trees, and the fruits were smaller and with pale ground colour and good pinkish-red flush. The flesh was white and hard, and the flavour sweet and much less acid than that of the comparable fruits. Picking also took place on October 14th. No storage breakdown was found during the test.

Discussion of results.

The results of the biological tests show consistently a higher antiscorbutic activity in the case of the apples in which the nitrogen is lowered whether it be by cultural means or by bark-ringing (Tables I and II). This increased activity although not so very marked in some Bramley's Seedling samples, namely those of 1929 and 1931, falls definitely outside the limit of the experimental error of the prophylactic method in the other samples of this variety. Quite possibly lower test doses might have revealed a more marked difference. Considering that the results obtained during several years have pointed always in the same direction little doubt can be entertained concerning their significance. Moreover, the lowering of the nitrogen content in the King Edward apples by cultural treatment or by ringing raised the vitamin C content of the fruit as much as 1·5 to 2 times. It is quite likely that the Bramley's Seedling variety being antiscorbutically one of the most active, possesses almost the maximum potency possible in the apple. The chemical data and the general biological characteristics of the apples tested leave no doubt that the differences observed in the nitrogen content are also significant.

Table II.

Dose g. daily	Bramley's Seedling 1929. High nitrogen	Bramley's Seedling 1929. Low nitrogen	King Edward VII 1929. High nitrogen	King Edward VII 1929. Low nitrogen	Bramley's Seedling 1930 not ringed. High nitrogen	Bramley's Seedling 1930 ringed. Low nitrogen
3	+ + + ⊥	+ + + +	No protection	⊥	+ + +	+ + + +
5	+ + + +	+ + + +	⊥	+ ⊥	+ + + +	+ + + +
10	+ + + +	+ + + +	+ + ⊥	+ + + ⊥	+ + + +	+ + + +
20	*	*	+ + + +	+ + + +	+ + + +	+ + + +

	Bramley's Seedling 1930. High nitrogen	Bramley's Seedling 1930. Low nitrogen	Bramley's Seedling 1931 not ringed. High nitrogen	Bramley's Seedling 1931 ringed. Low nitrogen	King Edward VII 1932 not ringed. High nitrogen	King Edward VII 1932 ringed. Low nitrogen
3	+ + +	+ + + +	+ + + ⊥	+ + + +	⊥	+ ⊥
5	+ + + +	+ + + +	+ + + +	+ + + +	+ ⊥	+ + + ⊥
10	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
20	+ + + +	+ + + +	+ + + +	+ + + +	Not tested	Not tested

* Test not finished but fully protected for 55–60 days.

Although the nitrogen content and the activity of these apples were always inversely related, it is evident that there was no strict proportionality between them. When it is borne in mind that the nitrogen fraction of the apple consists in all probability of various nitrogenous compounds this would not be expected.

A survey of other analytical data shows that acidity, sucrose and ash constituents show no correlation with the vitamin activity. On the other hand the decreases in nitrogen content in all cases are also associated with increases in the ratio total sugars/nitrogen, the increases being due in all instances both to lower values for nitrogen and higher values for total sugars. It is difficult at this stage of the inquiry to interpret the significance of these observations, but it is obvious that a connection cannot be excluded between some nitrogenous constituent or constituents and vitamin C in the metabolism of the apple.

We are indebted to Miss M. F. Bracewell and Mrs K. M. Balfour for helping us with the tests in this investigation.

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XCII. THE VITAMIN B COMPLEX AND HIGH PROTEIN DIETS.

By FRANCIS THOMAS GARNET PRUNTY
and MARGARET HONORA ROSCOE[1].

From the Division of Nutrition, Lister Institute, London.

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Drummond, Crowden and Hill [1922] showed that rats and kittens were retarded in growth when their diets contained large proportions of protein. This effect was again observed by Reader and Drummond [1925], who used three levels of protein intake and found that young rats grew progressively less as the protein in the diet was increased. The same authors showed [1926] that by increasing the vitamin B content the effect of the high protein diet could be counteracted and considered that this fact confirmed the theory put forward by Hartwell [1924; 1925] that the vitamin B requirements are dependent on the protein intake.

Hartwell's experiments were carried out with young suckling rats, in which spasms developed when the protein of the mothers' diet was increased, unless additional yeast extract was given. They are not therefore comparable with the above experiments or with those reported in this paper, which deal with the growth of young weaned rats, and it is not proposed to discuss them here.

Hassan and Drummond [1927] carried the matter further by showing that additional amounts of an autoclaved yeast extract were as effective in restoring growth as an unheated yeast extract. The differentiation between vitamins B_1 and B_2 had not at that time been completed, but the result of this experiment has since been taken as evidence that the more heat-stable vitamin B_2 is the factor in the vitamin B complex which counteracts the unfavourable effect of a high proportion of protein in the diet.

In addition to the effect of high protein diets on growth, their effect on the kidney has been studied. Polyvogt, McCollum and Simmonds [1923] found degenerative changes in the kidneys of rats receiving diets containing 31 to 41 % of protein. This has not been confirmed by later workers. Reader and Drummond [1925; 1926] and Hassan and Drummond [1927] observed hypertrophy of the kidneys but no degenerative changes, although their animals in some cases received as much as 90 % protein. Reader and Drummond [1926] claimed to have shown that this hypertrophy did not occur in rats which received additional amounts of vitamin B and grew normally, but only in those in which growth was retarded. It thus appeared that a balance must be maintained between the protein intake and the vitamin B_2 intake if normal growth and kidney development are to take place.

Since this work was carried out before the differentiation of vitamins B_1 and B_2 had been made, it was thought advisable to reinvestigate the question. The following experiments were therefore carried out.

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[1] Beit Memorial Fellow.

EXPERIMENTAL.

Exp. 1. *The effect on rats of a high protein diet at various levels of vitamin B_2 intake, vitamins B_1 and B_2 being given as separate daily doses.*

Method. Two levels of protein intake were employed, 20 and 70 %. The diets had equal calorie values and were constituted as follows:

	%	%
Caseinogen ("Casumen"*)	20	70
Rice starch	60	10
Salt mixture†	5	5
Hardened cottonseed oil	15	15

* A commercial caseinogen not specially purified.
† Salt mixture No. 185 [McCollum, Simmonds and Pitz, 1917].

The diets were mixed with an equal amount of water and cooked in a steamer for 3 to 5 hours. Cod-liver oil was given as a separate daily dose (0·05 cc.).

Vitamin B_1 was given as an antineuritic concentrate from yeast [Peters, 1924; Kinnersley and Peters, 1925], vitamin B_2 as an autoclaved acetic acid extract of yeast (1 cc. ≡ 1 g. dry yeast) [Roscoe, 1930].

Young 40 g. rats previously deprived of the B vitamins for 1 week were used, and the effects of the two diets observed at different levels of vitamin B_2 intake with respect to growth, kidney weight and incidence of dermatitis characteristic of vitamin B_2 deficiency. The food intake was also noted.

Vitamins B_1 and B_2 were given as separate daily doses, all rats receiving adequate vitamin B_1. The two diets with different percentages of protein were administered to two parallel series of rats of which group 1 received no vitamin B_2 and groups 2, 3 and 4 increasing amounts; the dose received by group 4 was more than adequate for normal growth. The rats were observed for 5 weeks, with the exception of those receiving no vitamin B_2, which were kept for 15 weeks or until dermatitis developed.

Results.

(i) *Growth* (Table I). At a given intake of vitamin B_2 growth was the same whether the percentage of protein in the diet was 20 or 70. A slight difference occurred in group 4, but this was probably not significant.

(ii) *Dermatitis.* Six of the eight rats receiving no vitamin B_2 (group 1) exhibited dermatitis. Three of these had received the diet with 20 % protein and showed symptoms in 7½, 8 and 9½ weeks, and three had received the diet with 70 % protein, showing symptoms in 7½, 7½ and 13 weeks. The amount of protein in the diet did not therefore appear to influence in any way the incidence of dermatitis.

(iii) *Kidney weight.* The kidneys of the rats in groups 2, 3 and 4, receiving different amounts of vitamin B_2, were weighed at the end of the experimental period of 5 weeks (Table II). Those of the rats receiving 70 % protein were in all cases larger than those of the rats receiving 20 % protein. This hypertrophy did not decrease with increasing amounts of vitamin B_2. It was, in fact, found, as may be seen by reference to Table II, to be roughly proportional to the increase in protein consumption, whatever the dose of vitamin B_2.

(iv) *Food intake.* In Table II are also shown the average amounts of food consumed by each group of rats during the 5 weeks of the experiment. The rats

Table I. *The growth of rats receiving diets containing 20 or 70 °/₀ protein at different levels of vitamin B_2 intake.*

	Vitamin B_2 daily dose cc.	Increase in weight in 5 weeks g.					
		20 % protein diet			70 % protein diet		
		Rat		Average	Rat		Average
1	0·0	676 ♂	18	22	680 ♂	29	21
		692 ♂	21		696 ♂	18	
		677 ♀	15		681 ♀	22	
		693 ♀	36		697 ♀	15	
2	0·15	729 ♂	47	42	733 ♂	59	43
		730 ♂	38		734 ♂	39	
		731 ♀	44		735 ♀	45	
		732 ♀	40		736 ♀	40	
3	0·5	684 ♂	83	76	698 ♂	87	78
		685 ♀	72		699 ♂	87	
		695 ♀	73		688 ♀	73	
					689 ♀	66	
4	1·75	678 ♂	133	111	682 ♂	100	92
		686 ♂	129		690 ♂	100	
		679 ♀	89		683 ♀	97	
		687 ♀	95		691 ♀	71	

Table II. *Kidney weights and food intakes of rats fed for 5 weeks on diets containing 20 or 70 °/₀ protein and on different amounts of vitamin B_2.*

Averages of 4 rats.

Dose of vitamin B_2	(1) 0·0 cc.		(2) 0·15 cc.		(3) 0·5 cc.		(4) 1·75 cc.	
	g.	% increase	g.	% increase	g.	% increase	g.	% increase
Kidney weight g.:								
20 % protein	—	—	1·068	33	1·535	17	1·724	27
70 % protein	—		1·420		1·790		2·176	
$\frac{\text{Kidney weight}}{\text{100 g. body weight}}$								
20 % protein	—	—	1·209	—	1·220	—	1·110	—
70 % protein	—		1·540		1·396		1·476	
Food intake g. dry weight in 5 weeks								
20 % protein	202	−27	201	−14	399	−34	431	−27
70 % protein	147		173		263		316	
Protein intake g. in 5 weeks								
20 % protein	40	157	40	202	80	130	86	156
70 % protein	103		121		184		220	
$\frac{\text{\% increase in kidney weight}}{\text{\% increase in protein intake}} \times 100$	—		16		13		17	

fed on the 70 % protein in every case ate considerably less than those on 20 %, but notwithstanding they grew as well.

This, in conjunction with the fact that in the experiments of Drummond *et al.* [1922], Reader and Drummond [1925; 1926] and Hassan and Drummond [1927] the vitamin B complex was given as a percentage of the diet, suggested a possible reason for the fact that the present experiments fail to confirm the observations of those workers. A second experiment was therefore carried out in which vitamins B_1 and B_2 were given as a percentage of the diet incorporated in it.

Exp. 2. *The effect on rats of a high protein diet, when vitamins B_1 and B_2 are incorporated as a percentage of the diet.*

Method. The diets were the same as those in Exp. 1 with the exception that a yeast extract containing both vitamins B_1 and B_2 was added, in the proportion of 10 cc. per 100 g. dry weight of diet. (This yeast extract was prepared in the same manner as that used as a source of vitamin B_2 but was not autoclaved.)

Two groups of four rats of one litter were fed on each diet for 5 weeks.

Results. In Table III are shown the total weight increases of these rats, the amounts of food which they ate during the 5 weeks of the experiment, and, calculated from this, the average amounts of yeast extract consumed *per diem*.

Table III. *The growth of rats receiving diets containing 20 or 70 % protein and 10 % yeast extract to supply vitamins B_1 and B_2.*

	20 % protein diet					70 % protein diet				
Rat	824 ♂	825 ♀	826 ♀	827 ♀	Av.	828 ♂	829 ♂	830 ♀	831 ♀	Av.
Increase in weight in 5 weeks g.	100	86	88	92	91	52	58	34	33	44
Food eaten in 5 weeks g. dry weight	285	298	297	325	301	175	159	145	162	160
Yeast extract average *per diem* cc.	0·81	0·85	0·85	0·93	0·85	0·50	0·45	0·41	0·46	0·45

The rats receiving the high protein diet showed a much smaller weight increase than those receiving the low protein diet, but they also ate much less and thus obtained smaller quantities of the yeast extract incorporated in the food. It is calculated that the two groups actually received 0·85 and 0·45 cc. respectively of this yeast extract daily, and the weight increase was of the order that would have been expected in such circumstances.

DISCUSSION.

These experiments show that although rats fed on a high protein diet grew less well than did those on a low protein diet, this only occurred if some growth-promoting factor was supplied as a percentage incorporated in the diet. Reduced intake was a constant feature with the high protein diets and thus produced a reduced intake of the B vitamins when these were incorporated in the diet. This is not in agreement with the data given by Reader and Drummond [1925]. Here the food intake of the rats on the high protein diets (45 and 90 % caseinogen) was recorded as being greater than that on a 20 % protein diet. In a later paper however [Hassan and Drummond, 1927], the food intake was recorded for rats on 70 and 20 % caseinogen diets and was definitely lower on the former (average of 39 against 58 g. in 4 days). The respective amounts of yeast extract consumed were thus 0·4 and 0·6 g. *per diem*. In this case, therefore, it appears probable that it was the reduced intake of yeast extract that was limiting growth.

In the same paper [Hassan and Drummond, 1927] it was also shown that addition of extra amounts of the heat-stable fraction of the vitamin B complex (B_2) restored growth. Vitamins B_1 and B_2 were supplied together in the yeast extract used (marmite) and this extract has since been shown to supply vitamin B_1 in doses smaller than suffice for vitamin B_2. The deficiency of vitamin B_2 thus became apparent before that of vitamin B_1. This was also the case in Exp. 2 of the present series where the amounts of the yeast extract consumed in the low and high protein groups, 0·85 and 0·45 cc. *per diem*, contained sufficient vitamin B_1 for normal growth while vitamin B_2 was present in limiting amounts.

It is however possible that the supply of any factor necessary for growth or maintenance, when incorporated as a percentage of the food, might become insufficient when a high protein diet, of which the intake is low, is being fed. In this connection it may be noted that Drummond, Crowden and Hill [1922], giving vitamins A and D as shark-liver oil, 2 to 3 % of the diet, noted beading of the ribs, lameness and symptoms of joint trouble in rats and kittens on high protein diets.

The fact that in Exp. 1 the food intake was higher on the 20 % protein diet than on the 70 % protein, suggested, at first, that 20 % protein (in the form of caseinogen) is below the optimum, so that the rats on this diet ate more in order to acquire sufficient protein. This does not seem probable as Osborne and Mendel [1915] have shown that when rats received a diet containing a sub-optimum percentage of protein they ate less, rather than more, of that diet. These authors also showed that good growth occurred when the diet contained 15 % caseinogen and that increasing the percentage to 18 did not result in more growth; 15 % caseinogen therefore was the optimum amount. Their rats receiving the 18 % caseinogen diet ate more of the diet than did those receiving 15 %, although growing to the same extent. It therefore appears that the food consumption of these rats was more than was necessary and this, it is suggested, was the explanation of the high food intake of the rats in the present experiments receiving the 20 % caseinogen diet. They ate more food than they needed, while the rats fed on the 70 % protein diet, possibly owing to the unpalatability of the diet, ate enough but no more.

The results obtained in Exp. 1 also prove that the intake of vitamin B_2 directly affected the rate of weight increase independently of any effect it may have had on appetite. Growth was constant for any given dose of vitamin B_2, whereas the calorie intake varied with the food consumption within wide limits, according to whether the diet had a high or low protein content. Also the food consumption of the rats fed on the 20 % protein diet receiving no vitamin B_2 (group 1) was the same as that of those receiving the smallest dose of vitamin B_2 (group 2), but the rats with vitamin B_2 grew twice as well as those with none.

That vitamin B_1 has a direct effect on growth has already been demonstrated by another method by Sure, Kik and Smith (1932).

SUMMARY.

1. No relation has been found between the protein intake and the vitamin B_2 requirements of rats.

2. Excess protein in the diet had an indirect depressing effect on growth, since it caused a reduced food intake. This resulted in a lowered vitamin intake when the vitamins were incorporated as a percentage of the diet.

3. The supply of vitamin B_2 had a direct effect on growth independent of any effect on appetite.

4. A high protein diet did not affect the incidence of the dermatitis characteristic of vitamin B_2 deficiency.

5. Hypertrophy of the kidneys of rats on a high protein diet was independent of the vitamin B_2 supplied.

Our thanks are due to Miss E. M. Hume for advice and criticism, and to the Lister Institute for hospitality.

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XCIII. GLYCOGEN FORMATION IN THE FOWL.

BY ARTHUR RAYMOND GORDON EMSLIE
AND KATHLEEN MARY HENRY.
Rowett Research Institute, Aberdeen.

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THE rate of glycogen formation in the liver of the rat after ingestion of various substances has been studied by many observers. It has been shown that, compared with other substances, the hexoses are more readily converted into glycogen, the order for the three commonest monosaccharides in this regard being glucose > fructose > galactose. It was considered of interest to carry out similar experiments in the fowl to ascertain whether the carbohydrate metabolism in birds was different from that in the mammal.

The research was begun by one of us (A. R. G. E.) in Toronto in 1931, the experiments taking the form of comparisons between the rate of deposition of liver-glycogen after ingestion of glucose and of several 3-carbon compounds. At Aberdeen the scope of the investigation was modified, so as to bring it into line with research being conducted there on the absorption and metabolism of carbohydrates in fowls. The object of the Aberdeen experiments was to determine the relationship between the rates of absorption and of glycogen formation after ingestion of various carbohydrates.

Toronto experiments (by A. R. G. E.).

The substances investigated were glucose, dihydroxyacetone, alanine, glycerol, sodium *dl*-lactate and sodium pyruvate. Barred Plymouth Rock chicks, weighing 100–200 g., and reared under the same conditions, were used[1]. Chicks were selected for greater uniformity and because it was thought that, owing to more active metabolism, changes in the liver-glycogen would be more marked in the small birds. In addition, the livers of these chicks weighed about 5 g., a convenient amount for analysis. The procedure employed was similar to that of Cori and Cori [1926]. After a preliminary fast overnight, followed by access to the food hoppers for half an hour, the birds were fasted 24 hours and given, by stomach tube into the gizzard, 5 cc. of a 50 % aqueous solution of the substance to be tested, warmed to 38° and neutralised, if necessary, with NaOH. We found that if more than 5 cc. of fluid were given, there was danger of regurgitation into the crop. For comparison with the work of Cori and others on rats, all substances were administered in 50 % solution with the exception of alanine, which was given in 30 % solution. Glycogen was determined in the liver and pectoral muscles by Irving's method [1928], the Shaffer-Hartman method [1920] being used for estimating the sugar in the hydrolysate. The pectoral muscles were chosen, as they are easily accessible and quite large, even in a small chick. At the same time it was realised that a change in the percentage of glycogen in these muscles might not be comparable with Cori's results for the glycogen of "the rest of the body." Birds of both sexes were used because, as the following control experiment showed, there was no sex difference in the initial liver- or muscle-glycogen.

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[1] These birds were kindly supplied by the Poultry Department, Guelph, Ontario.

15 ♂ av. liver-glycogen, 0·126 % ±0·029; muscle glycogen, 0·186 % ±0·114
15 ♀ „ 0·136 % ±0·045; „ 0·192 % ±0·100

All chicks used in the investigation were fasted for 24 hours before the administration of the test substance; as the data given below indicate, the percentage of liver-glycogen of chicks fasted 48 hours was somewhat higher than that of those fasted 24 hours. Furthermore, the vitality of the birds was considerably lowered by a fast of 48 hours.

6 birds fasted 24 hrs., av. liver-glycogen, 0·149 % ±0·059; muscle-glycogen, 0·294 % ±0·106
6 „ 48 „ „ 0·229 % ±0·033; „ 0·314 % ±0·060

Results.

Liver-glycogen. The average results are shown in Fig. 1. It can be seen that the glucose curve, so far as it goes, is of the same general type as that found by Cori and Cori [1926] in the rat. The curve for dihydroxyacetone, on the other hand, in its similarity to the smoothed glucose curve, differs from what might be expected from the figures of Cori and Cori [1928] for absorption and liver-glycogen deposition after ingestion of the triose in the rat, and suggests that the absorption of this sugar is more rapid in the chicken. Alanine gave a gradual increase in the liver-glycogen, the value reaching its maximum (1·5 %) at 4·5 hours, a somewhat higher figure than that found by Wilson and Lewis [1929] in the rat. Glycerol, on the other hand, gave only a slight increase, the percentage obtained by Catron and Lewis [1929] in rats being almost three times as great. Neither sodium pyruvate nor sodium *dl*-lactate gave rise to

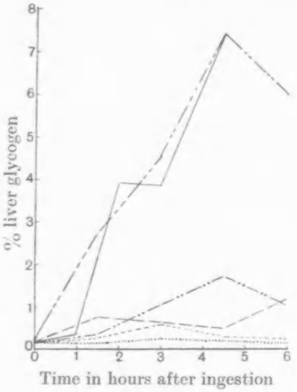

Fig. 1.

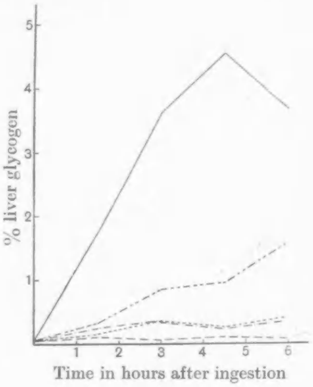

Fig. 2.

Fig. 1. Average liver-glycogen, Toronto experiments, after ingestion of: Glucose ——; Dihydroxyacetone — — — —; Alanine —••—; Glycerol — — —; Na lactate - - - -; Na pyruvate ······.

Fig. 2. Average liver-glycogen, Aberdeen experiments, after ingestion of: Glucose ——; Galactose — — — —; Lactose - - - -; Starch —•—; Egg-white — — —.

noticeable increases in liver-glycogen. Glycerol, and particularly sodium pyruvate, were found to be toxic, so that some birds died before the end of the experiment.

Muscle-glycogen. There was no increase in the percentage of glycogen in the pectoral muscles after feeding any of the trioses. Some of them, indeed, appeared to cause a fall in glycogen percentage. With glucose there was an increase, the maximum (0·8 %) occurring 2 hours after ingestion. It is of interest to note that dihydroxyacetone, which gave such large increases in liver-glycogen, caused no change in the glycogen of the pectoral muscles.

Aberdeen experiments (by A. R. G. E. and K. M. H.).

The substances tested were glucose, galactose, lactose, starch and egg-white. They were given in 50 % aqueous solution or suspension (starch), except egg-white which was dissolved in 0·9 % NaCl. The procedure was similar to that employed in the Toronto experiments, except that S.C. White Leghorn chicks were used, and glycogen determined by the method of Cori and Cori [1926] on "the rest of the body" after skinning and eviscerating the animals, and cutting off the heads, tips of wings and legs at the tibio-tarsometatarsal joint. The bodies were not frozen; preliminary experiments, cited below, showed that, under the experimental conditions, there was no marked difference in the initial levels of body-glycogen of birds which were not cooled, cooled in brine at − 10° or frozen with CO_2.

No cooling: av. body-glycogen	0·064 % ±0·012
Brine, − 10°	,,	0·091 % ±0·013
CO_2	,,	0·090 % ±0·006

The average liver-glycogen of 8 control chicks was 0·049 %, considerably less than in the Toronto experiments.

The rates of absorption of glucose, galactose and lactose were determined, but owing to technical difficulties we were unable to do this with starch. Preliminary experiments showed that the whole digestive tract of chickens starved for 24 hours had no appreciable amounts of reducing substances, 1·7 mg. on an average in 12 birds. The recovery of glucose from 4 chickens killed immediately after feeding varied from 86 to 96 % with an average of 90 %; but, as will be shown later, the absorption of glucose was very rapid, and this might account, in part at least, for the poor percentage recovery. During the period of absorption the chicks were placed on filter-papers and at the conclusion of the experiment the washings from these were added to those of the digestive tract. In only a few instances, as after galactose feeding, was there any diarrhoea. Lactose was determined by adsorption on to yeast before and after hydrolysis. Blood-sugar determinations (Hagedorn-Jensen) were made in a few instances only, because of the rapidity with which the blood clotted.

Results.

Absorption. Table I shows the absorption as determined at different periods after ingestion. Comparing these results with Cori's [1925], it is seen that chickens differ from rats in that the rate of absorption of the sugars is not

Table I. *Absorption coefficients (g./100 g. live wt./hour).*

Substance	1·5 hrs.	3 hrs.	4·5 hrs.	6 hrs.
Glucose	0·655	0·422	0·341	0·245
Galactose	0·645	0·365	0·275	0·216
Lactose	0·355	0·175	0·145	0·115

constant between 1·5 and 6 hours, but falls considerably, especially during the later periods. Another difference is that galactose is absorbed somewhat more slowly than glucose. Lactose, as was expected, has a lower A.C. (absorption coefficient) than either glucose or galactose. At 1·5 hours the relative rates of absorption are: glucose 100, galactose 98, lactose 54. At the other periods, however, the order is glucose 100, galactose 85, lactose 43.

Table II. *Average blood-sugar (g./100 cc.).*

Substance fed	0 hrs.	1·5 hrs.	3 hrs.	4·5 hrs.	6 hrs.
Glucose	0·200	0·322	0·305	0·202	0·197
Galactose	0·200	0·308	0·284	0·295	—
Lactose	0·200	0·216	0·200	0·174	0·197
Starch	0·200	—	0·230	0·213	0·212
Egg-white	0·200	0·209	0·206	0·205	0·189

While the values given in Table II are of interest in indicating the more rapid return of the blood-sugar to normal after feeding glucose than after galactose, and that it was not affected by a protein such as egg-white, they should be considered with caution because they are averages of single determinations on different individuals, and as MacOwan and Magee [1931] and others have shown, there is considerable individual variation in adult fowls.

Liver-glycogen. The average results are shown in Fig. 2. Glucose caused a marked increase in liver-glycogen; this was, however, considerably less than in the Toronto experiments. In comparison with glucose, very little glycogen was deposited in the liver after galactose ingestion, still less after lactose or starch and none after egg-white. These results are somewhat surprising in view of the relatively rapid rates of absorption of the sugars. Harding and Moberley [1930] have shown that after ingestion of galactose by human beings the sugar is excreted in the urine, and Cori [1925] found that galactose was largely excreted in the urine of the rats in his experiments. Loss in the urine, however, could not account for the disposal of the galactose in our birds; for the average amount of galactose recovered from the mixed excreta of the birds killed at the different periods was as follows: 1·5 hrs., 0·439 g.; 3 hrs., 0·454 g.; 4·5 hrs., 0·481 g.; 6 hrs., 0·450 g. This indicates that although absorption continued, very little galactose was excreted after 1·5 hrs.

Body-glycogen. Glucose was the only substance which caused a marked increase in body-glycogen, the value rising from 0·064 % to a maximum of 0·42 % at 3 hrs. and falling to 0·33 % at 6 hrs. The muscle-glycogen of the chicks fed with the other substances was apparently not affected, the changes in the percentage of body-glycogen being insignificant.

Table III. *Percentage of sugar absorbed which was deposited as liver-glycogen.*

	Hours after ingestion			
Sugar fed	1·5	3	4·5	6
Glucose	4·71	2·92	11·84	9·03
Galactose	0·87	2·57	2·46	3·85
Lactose	0·83	0·39	0·10	0·16

Table III shows the percentage of sugar absorbed which was recovered as liver-glycogen (calculations being based on the figures obtained for the control chicks given previously).

DISCUSSION.

Wilson and Lewis [1929] point out that large experimental animals are unsuitable for studies of glycogen formation because they often have considerable reserves of glycogen even after fasts of several days. From the figures given for the percentage of glycogen in the livers and bodies of chickens starved for 24 hours, however, it appears that birds of this weight are as suitable in this respect as the rat; for in starvation the glycogen stores of the liver are rapidly depleted to a fairly constant level. It seems from a comparison of the data obtained in Toronto and in Aberdeen, that both liver- and body-glycogen may vary according to breed and environment.

In the Toronto experiments the liver-glycogen was slightly, but definitely, higher after 48 hours than after 24 hours of starvation. This fact may possibly be related to the observation made by MacOwan and Magee [1931] and by Henry, Macdonald and Magee [1933] that the blood-sugar of the fasting fowl shows unaccountable fluctuations, rising to a peak at about 72 or 96 hours after an initial fall. It is possible that this rise occurs earlier in the chicken and may be accompanied by a corresponding rise in the liver-glycogen.

From a study of the rates of absorption of glucose, galactose and lactose, birds seem to differ somewhat from the rat. While the question has not been studied sufficiently in detail, it appears that in young chickens, the absorption of galactose and more especially of glucose is not independent of the amount present in the digestive tract, as Cori [1925] has shown for the rat. Thus, in the Aberdeen experiments the A.C. for glucose fell from 0·655 g. at 1·5 hours when about 66 % of the sugar had been absorbed, to 0·422 g. at 3 hours when about 90 % of the sugar had been absorbed. Absorption is also considerably more rapid in the chicken than in the rat, as a comparison of our A.C. values with those of other workers will show. Another difference should also be noted, *viz.* that galactose is absorbed by the chick less rapidly than glucose, whereas Cori found the opposite in the rat.

From Fig. 2 it can be seen that the chick can rapidly deposit glycogen in its liver from absorbed glucose. Galactose and lactose, on the other hand, are not utilised in the same way, the liver- and muscle-glycogen during the absorption of these sugars showing only small increases. In view of the results published by MacOwan and Magee [1931] and Henry, Macdonald and Magee [1933] on the effect of feeding finely ground starch-rich foods on the blood-sugar level of adult birds, it is interesting to note that, with the birds in our experiments, the blood-sugar did not appear to increase, nor was there an appreciable deposition of glycogen in the liver or body as a result of starch feeding. This may be due to slower digestion of starch or more rapid utilisation of absorbed sugar by young chicks than by adult fowls.

It appears from the Toronto experiments that, if the rates of absorption of the two sugars are similar, dihydroxyacetone can be formed into liver-glycogen in the chick as readily as can glucose. It is curious, however, that there was no formation of glycogen in the pectoral muscles, and there is little reason to believe that there was in the other muscles. Mostkowski [1911], however, found an increase in the percentage of muscle-glycogen after feeding as much as 60 g. of the triose to adult hens previously starved for 4 or 5 days. The reason for these divergent results is not clear; they may be related to the differences in age of the birds used in the respective investigations.

Pyruvic acid was cited by Lusk [1928] as a possible intermediary in the conversion of alanine into glucose, but, in view of the definite increase in liver-glycogen after the feeding of the amino-acid to our birds and the negative results obtained with sodium pyruvate, it is probable that pyruvic acid is not an intermediary in chickens. The experiments do not deny this possibility, however, for the pyruvic acid may have been metabolised in some other way. It is of interest that in the experiment where starch was given, there was little deposition of glycogen. Glycerol, also, while being formed into glycogen readily in the rat, was not utilised in this way in our experiments.

SUMMARY.

Experiments were conducted on chickens weighing 100–200 g. to find the rates of absorption of glucose, galactose and lactose and of glycogen formation after the ingestion of these sugars, of several 3-C compounds and of starch and egg-white. The relative rates of absorption were in the following order: glucose > galactose > lactose, and they fell gradually between 1·5 and 6 hours after ingestion. The rate of liver-glycogen formation was in the order glucose = dihydroxyacetone > alanine > glycerol > Na lactate > Na pyruvate in the Toronto experiments, and glucose > galactose > lactose = starch > egg-white in the Aberdeen experiments. Muscle-glycogen was increased by glucose ingestion but not by that of any of the other substances.

The Toronto experiments were conducted in the Department of Biochemistry at the suggestion of Dr H. D. Kay, and were made possible through grants from the Department of Biology and the Ontario Agricultural College. At the Rowett Institute we have been indebted to Prof. J. J. R. Macleod and Dr H. E. Magee for advice and criticism, and to the Medical Research Council for a grant to one of us (K. M. H.).

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XCIV. THE DIALYSIS OF MILK.

THE DISTRIBUTION OF PHOSPHORUS.

By LESLIE HERBERT LAMPITT and JOHN HERBERT BUSHILL.

From the Laboratories of Messrs J. Lyons and Co., Ltd., Kensington, W. 14.

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NOTWITHSTANDING the vast amount of work which has been carried out in attempts to define the constituent compounds of normal cow's milk, no very clear or even comprehensive statement can yet be made. A gross division has been effected, or rather perhaps a number of compounds have been separated from milk, but in the majority of cases chemical methods have been employed which, in themselves, must have brought about interaction between the reagents used and the constituents of the milk. The problem is, of course, complicated by the fact that milk is not a true solution, but a mixture of substances in true solution with others dispersed through the medium as colloids: this introduces the question of adsorption on surfaces and also the possibility of change, according to conditions, of some substances from true solution to colloidal dispersion, or *vice versa*.

It is suggested that until some method be devised whereby the constituents of milk can be separated by physical means, or without the use of chemicals which themselves react with the milk, no definite hypothesis concerning the composition of milk or even of any of its constituent parts such as caseinogen, can be held to be free from serious criticism. This fact has been realised by some workers, and Lenstrup [1926] has stated that in the separation of the acid-soluble phosphorus compounds the difficulty is overcome by the use of picric acid. The constituents of milk are, however, so numerous, so little understood and some of them certainly so easily changed in composition that it appears that dialysis and ultrafiltration are methods least open to criticism from the point of view of outside interference. It was with the object of using such physical methods that the present investigation was undertaken and the continuous dialysis apparatus developed.

It is obvious that the progress of the dialysis of milk can be followed by tracing the changes in the distribution of any of the numerous dialysable constituents. In this particular study, phosphorus (as inorganic and organic) was chosen, but when it was desired to compare the results obtained with those of other workers, it was found that there was no general consensus of opinion regarding the distribution of this element; Table I demonstrates this point. It appears that there are two main reasons for this lack of conformity, namely, variation in the milk and variation in the apparatus used.

(*a*) *Milk*. It is self-evident that if there is any variation in the natural phosphorus content of milk, it will be almost impossible to express in general terms the amount—both inorganic and organic—dialysable under any series of conditions. Moreover, in order to make comparison possible, all results should be referred to a common basis, such as the non-fatty solids content of the milk. The majority of recorded analyses have not been so calculated nor has the

Table I. *The phosphorus distribution in milk reported by other workers.*

Reference	Method of separation	Form of phosphorus	Amount of phosphorus found in milk %	Amount of phosphorus found in serum %
Van Slyke and Bosworth (1915)	Pasteur-Chamberland filter	Inorg.	0·096, 0·087	0·067, 0·056
	Separated milk. $CHCl_3$ extracted. (Chloroform as preservative)	Org.	0·029 (2 samples)	Nil — (54 % of total)
Van Slyke and Bosworth (1915)	Pasteur-Chamberland filter	Total	0·087–0·131 (13 samples)	0·029–0·082 (27–64 % of total)
Van Slyke and Bosworth (1916)	Pasteur-Chamberland filter	Inorg.	0·083	0·043
	Separated milk. (No preservative)	Org.	0·021 (1 sample)	—
Bell (1925)	Pasteur-Chamberland filter	Total	—	0·028
	Separated milk. (No preservative)	—	—	—
György (1923)	Ultra-filtration through de Haen filter membranes	Inorg.	0·030–0·036	0·013–0·019 (40–50 % of total inorg. P)
Wardlaw (1914)	Dialysis (collodion membranes) 25 cc. water in 1000 cc. separated milk	Total	0·093–0·115 (7 samples)	0·033–0·067 (35–55 % of total)
Mattick and Hallett (1929)	Dialysis of milk 25 cc. (parchment thimbles) in 150 cc. of 3 % NaCl for 18 hours	Total	0·099–0·114 (11 samples)	0·030–0·038 (29–38 % of total)

composition of the milk used been quoted. A further complication is introduced by the fact that a number of investigators have used milk from individual cows in order to evaluate the possible effects of factors incidental to the animal itself, such, for example, as the lactation period. The effects of these complicating factors must be obviated before conclusions, deduced from any phosphorus distribution determinations, can be regarded as reliable; this end could be realised either by averaging a large number of individual determinations or by carrying out experiments on what may be called "average" milk. In order to achieve this object, separated bulked milk (or spray process powder made therefrom) has been used in the work now described. The idiosyncrasy of the cow has thereby been nullified, but the possible effect of seasonal variation has not yet been considered; it appears that this is not, however, of great importance. The difficulty with all milk experiments is the progressive change taking place on keeping, and therefore milk in the form of powder would be of great value, as experiments could be carried out and repeated on the same bulk without fear of alteration having occurred; but it would be first necessary to show that dialyses of a milk and of a solution of a milk powder made therefrom, would yield identical results. This has not yet been done, but for convenience of the comparison of the various methods of serum production, milk powder has been used in the majority of experiments now described.

(*b*) *Apparatus used.* In the past the variations in the means of obtaining sera have added still more to the difficulty of comparing the results. Consequently in the present study three methods of separating a serum have been compared: (i) a continuous complete dialysis method (involving ultrafiltration); (ii) dialysis with minimum equilibrium disturbance (called herein "static" dialysis) and (iii) ultrafiltration. In all cases collodion membranes were used. These membranes were hand-made, and it is realised that criticism of this fact might be raised, but tests have shown that the errors introduced by slight variations in the structure of the membrane, made according to the technique described, are not significant to the present argument.

EXPERIMENTAL.

(a) *Method of continuous dialysis.*

The apparatus used is shown in Figs. 1 and 2. It will be realised that certain particular requirements are satisfied: (*a*) The speed of dialysis is increased by continuous stirring and by applying a pressure[1] which induces ultrafiltration of the milk through the collodion sac. (*b*) The milk is maintained at a low

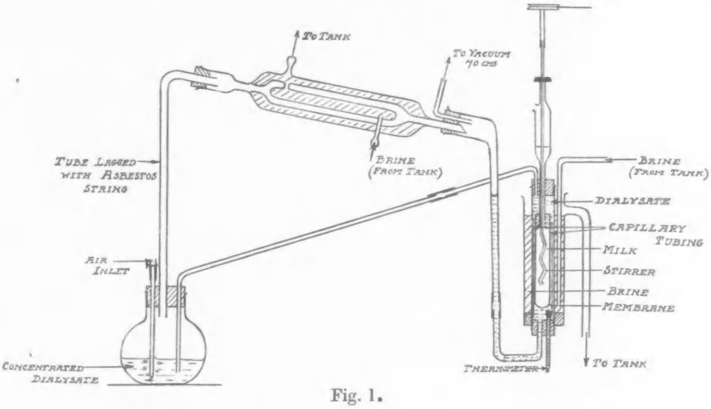
TO TANK
TO VACUUM 70 CMS
TUBE LAGGED WITH ASBESTOS STRING
BRINE (FROM TANK)
BRINE (FROM TANK)
DIALYSATE
CAPILLARY TUBING
MILK
STIRRER
BRINE
MEMBRANE
AIR INLET
CONCENTRATED DIALYSATE
THERMOMETER
TO TANK

Fig. 1.

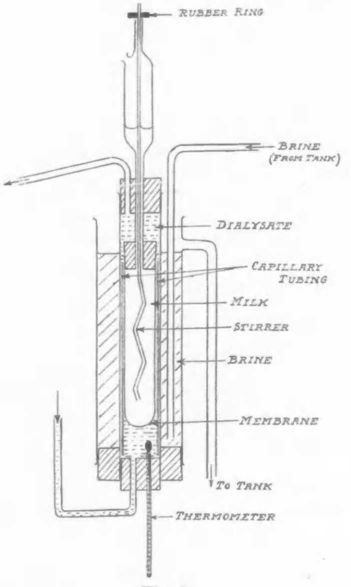
RUBBER RING
BRINE (FROM TANK)
DIALYSATE
CAPILLARY TUBING
MILK
STIRRER
BRINE
MEMBRANE
TO TANK
THERMOMETER

Fig. 2.

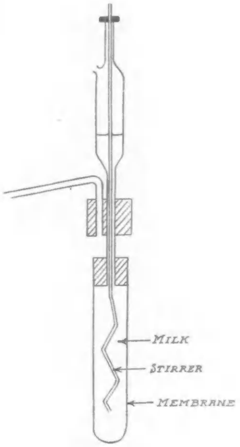
MILK
STIRRER
MEMBRANE

Fig. 2*a*.

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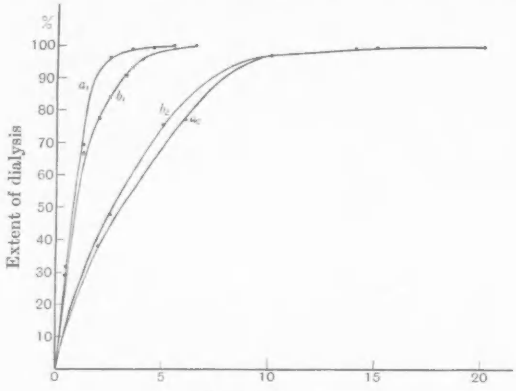
[1] Obtained by a reduced pressure outside the sac equivalent to 70 cm. of mercury.

temperature (0° to 5°). (*c*) The volume of water used is reduced to a minimum, thus enabling distilled water to be used, and in the process the dialysate is concentrated to convenient volume.

A point of major importance is that the speed and low temperature employed enable dialyses of milk to be conducted without the use of any preservative, as, at the temperature in question, very little change, induced by micro-organisms likely to be present, occurs. In all cases dialysis was continued until equilibrium had been attained (usually 40–48 hours).

The variation in the rates of dialysis using two different membranes is shown in Graph 1. Curve a_1 and curve b_1 represent experiments using sodium chloride solution (0·07 %), and curves a_2 and b_2 using a solution of separated milk powder[1] in which the reducing power[2] of the dialysate was used as the index of dialysis. In the experiments represented by curves a_1 and a_2 the same sac was used and similarly in the case of curves b_1 and b_2. The graph demonstrates that there is a satisfactory similarity between the two collodion thimbles used. Incidentally, it also shows that the dialysis of milk is very much slower than that of salt solution.



Graph 1.

The general procedure of a dialysis by this method is as follows.

The liquid (approx. 20 cc. of milk) to be dialysed is poured down the stirrer (a glass tube, see Figs. 1 and 2) thus enabling the air in the sac to escape into the cut-down pipette. Distilled water is added to bring the level of the liquid approximately to the position shown in the figure. The collodion sac is surrounded by distilled water in the vessel shown, and between it and the walls of this vessel are two capillary tubes to ensure free passage of water. This water is in continuous circulation, being delivered to the dialysis chamber by the condenser, and returned to the flask by means of the siphon. Since the distillation is carried out under reduced pressure, the temperature of the dialysate concentrating in the flask is between 45° and 50°. Moreover, the reduced pressure induces filtration of the milk through the membrane. The volume of milk is maintained by the addition of distilled water through the hole in the bulb of the pipette. Brine of suitable temperature is circulated through the condenser and the jacket of the dialysing unit during dialysis in order that the milk may be maintained below 5°.

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[1] In all cases the milk powder "solutions" have a concentration of about 10 % total solids, the actual concentration being checked by analysis. The milk powder used in this work was made by the "Milkal" spray process.

[2] In all cases a measure of the lactose has been obtained by using the ferricyanide reduction method of Hanes [1929] and when the reduction was small the method of Hagedorn and Jensen [1923]. The protein precipitant was trichloroacetic acid (2 %) which was neutralised in the filtrate prior to sugar determination.

At the termination of an experiment the dialysate in the flask may be recovered quantitatively (with careful washing of the bung and return pipe from the dialysing cell); the milk residue may be obtained likewise quantitatively by removing part of the dialysing unit Fig. 2 *a*, inverting so that the milk flows along the stem of the cut-down pipette and out through the hole in the side; the sac may be washed by the addition of water down the stirrer, the washings being recovered as described for milk.

In connection with the method of cooling, certain precautions should be taken, *e.g.* the brine must not cause blockage in the condenser due to freezing of the condensate, and, more important still, the temperature of the liquid surrounding the collodion sac must not be allowed to drop below about 2°, otherwise freezing may occur, with consequent puncturing of the sac.

The efficiency of the method using milk is illustrated by Table II.

Table II.

	Lactose (as % of the milk) removed in dialysis	Efficiency of dialysis calculated from lactose removed %
Dialysed 12 hrs.	4·20	90·0
Dialysed 24 hrs.	4·62	99·0
Dialysed 40 hrs.	4·65	99·6

The original milk contained 4·67 % lactose.

(*b*) *Method of static dialysis.*

The method was developed by Wardlaw [1914] who based it on the work of Rona and Michaelis [1909]; see also Moore and Bigland [1911].

The procedure by which the results now presented were obtained is as follows (see Fig. 3).

A collodion sac containing 25 cc. of distilled water is suspended in 900 cc. of milk contained in a 1-litre conical flask. The sac is supported by attaching it to the lower end of a piece of wide glass tubing and fixing the tubing in the neck of the flask so that the level of the water inside the sac is the same as that of the milk outside.

When equilibrium has been attained (after 48 hours at 0–5°) the milk, with regard to the dialysable portion, will have been diluted to the extent of the water in the thimble, that is, the effective volume becomes 925 cc. instead of the original 900 cc. Analysis of the liquid in the sac, when corrected for this dilution gives, therefore, the composition of the dialysable portion of the original milk. It may be noted that the liquid in the sac decreases in volume from 25 cc. to approximately 15 cc.

The statement of Wardlaw was confirmed that equilibrium is attained by this method in 16 hours but, since the continuous dialysis method requires 40–48 hours to come to completion, the static dialysis was continued for the same period in order that the figures obtained might be strictly comparable.

(*c*) *Method of ultrafiltration.*

The apparatus is shown in Fig. 4 and is a slight modification of the dialysis chamber used in the continuous method. Under these conditions approximately 5 cc. of filtrate were obtainable in 1–2 hours.

(*d*) *Method of determination of phosphorus.*

Inorganic phosphorus. A minimum amount of trichloroacetic acid, usually less than 0·5 % of the milk, is added to precipitate the organic matter, and the filtrate is used for the determination; the colorimetric method of Kuttner and Lichtenstein [1930] was adopted.

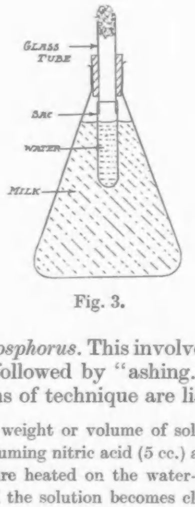

Fig. 3.

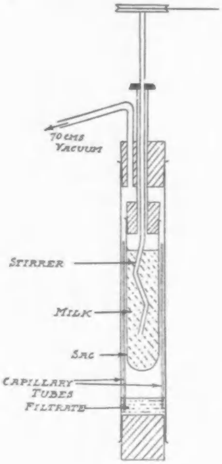

Fig. 4.

Total phosphorus. This involves the destruction of organic matter with fuming nitric acid followed by "ashing." It is well to describe this in some detail, as modifications of technique are liable to result in erratic figures.

A suitable weight or volume of solution is evaporated to dryness in a platinum dish on a water-bath. Fuming nitric acid (5 cc.) and approximately 0·01 g. of calcium carbonate are added and the mixture heated on the water-bath. The volume of the acid is maintained by further additions until the solution becomes clear, when the whole of the acid is allowed to evaporate off and the residue subsequently reduced to an ash.

This ash is dissolved in the smallest possible amount of 50 % trichloroacetic acid (usually 1 cc. is sufficient), and the solution transferred to a graduated flask and made to a convenient volume with distilled water. The amount of material to be taken and the ultimate dilution depend upon the phosphorus content of the material; using 5 cc. milk the final solution is made to 250 cc. The phosphorus determination is carried out by the same method as that used for the inorganic phosphorus.

(*e*) *Method of preparing collodion thimbles.*

The special collodion solution prepared by the British Drug Houses is diluted with half its volume of ether. A suitable volume of this solution is poured into a cut-down boiling-tube (of internal diameter slightly larger than the size of thimbles required) and the inside completely coated by careful rotation of the tube, finally allowing the excess liquid to drain away by inverting the tube. The rotation is continued for a further 5 minutes to allow some evaporation of the ether, and the tube is then carefully filled with 70 % alcohol and allowed to stand for 10 minutes. The alcohol is poured out and the tube and membrane placed in running water; after a short while the thimble may be disengaged and allowed to remain in the water until required (within 1 or 2 days). The thimbles are attached to suitable glass holders by sliding the latter into them for a distance of about half an inch and then drying the top of the thimble with a small gas flame. In this manner the top of the thimble is made to contract and hold firmly on the glass holder.

(*f*) *Comparison of static dialysis with ultrafiltration.*

A bulk of separated milk powder "solution" was prepared and eight lots of 900 cc. of this solution were each used for a "static" dialysis experiment at 0° to 5°. This number of dialyses was set up in order to provide sufficient material for the numerous determinations. After 2 days the solutions in the eight collodion sacs (now reduced from 25 cc. to approximately 15 cc.) were bulked as also were the milks remaining in the eight flasks. A sample of the bulked dialysate was removed for analysis, and of the remainder 15 cc. were replaced in each of six of the thimbles and the thimbles suspended in six flasks each containing 910 cc. of the bulked milk solution. This procedure was repeated on two further occasions.

At the intervals of time when the bulked static dialysates were sampled, samples of the original milk powder "solution," which had been stored at 0° to 5°, were subjected to ultrafiltration, and the ultrafiltrates analysed in a similar manner to the dialysates mentioned above.

This procedure ensured that any changes induced in the milk during storage would be the same in the two types of sera obtained. Typical results of two such experiments (Exps. 1 and 2) are recorded in Table III.

Table III. *Results calculated as percentage on non-fatty solids of original solution of spray-dried separated milk.*

Time of storage at 0° to 5° before treatment (days)	Exp. 1				Exp. 2			
	Static dialysate		Ultrafiltrate		Static dialysate		Ultrafiltrate	
	Total P	Inorg. P	Total P	Inorg. P	Total P	Inorg. P	Total P	Inorg. P
0	—	—	0·40	0·35	—	—	0·37	0·30
2	0·42	0·34	0·40	0·32	0·39	0·32	0·36	0·29
3	0·40	0·34	0·42	0·34	—	—	—	—
4	0·41	0·34	0·41	0·34	0·38	0·31	0·38	0·31
6	—	—	—	—	0·39	0·31	0·38	0·31
8	—	—	—	—	0·39	0·31	0·38	0·31
Average	0·41	0·34	0·41	0·34	0·39	0·31	0·37	0·30

A further somewhat similar series of experiments was carried out in order to compare different batches of spray-dried separated milk. In these experiments the static dialysis experiments were continued for 48 hours. The results are recorded in Table VII. Comment on this and on Table III is reserved for the general discussion of all the results.

(*g*) *Comparison of continuous dialysis with static dialysis.*

In this experiment (Exp. 3) Grade A.TT milk, separated at 32° and kept at 0° to 5°, was employed. After various times of storage, samples were subjected to continuous and to static dialysis, each process being allowed to proceed for 48 hours. In addition, the acid changes of the stored milk were determined [Lampitt and Bogod, 1930; 1932] by analysis of samples of the bulked milk at various times, with the results recorded in Table V; phosphorus analyses are given in Table IV.

Table IV. *Exp. 3. Percentage phosphorus calculated on non-fatty solids of original liquid separated milk.*

Time of storage before dialysing for 48 hours (days)	Phosphorus in combination as	Original milk	Continuous dialysis				Static dialysis	
			Residue (determined)	Dialysate (by difference)	Dialysate (determined)	Residue + dialysate (determined)	Residue (by difference)	Dialysate (determined)
0	Inorg.	0·71	0·11	0·60	0·51	0·62	0·30	0·42
	Org. (by diff.)	0·35	0·33	0·02	0·05	0·38	0·28	0·06
	Total	1·06	0·44	0·62	0·56	1·00	0·58	0·48
2	Inorg.	0·72	0·18	0·54	0·46	0·64	0·31	0·41
	Org. (by diff.)	0·36	0·26	0·11	0·01	0·26	0·29	0·07
	Total	1·08	0·44	0·65	0·47	0·90	0·60	0·48
10	Inorg.	0·72	0·15	0·56	0·56	0·71	0·28	0·44
	Org. (by diff.)	0·29	0·25	0·04	0·03	0·28	0·24	0·05
	Total	1·01	0·40	0·60	0·59	0·99	0·52	0·49
Average of 3 sets of results	Inorg.	0·72	0·15	0·57	0·51	0·65	0·30	0·42
	Org. (by diff.)	0·33	0·28	0·06	0·03	0·31	0·27	0·06
	Total	1·05	0·43	0·63	0·54	0·96	0·57	0·48

Table V. *The acidity of the separated milk of Exp. 3 during storage at 0–5°.*

Time of storage (days)	Total acidity ($N/4$ NaOH per 100 cc. milk) cc.	Lactic acid %	Citric acid %	Volatile acidity ($N/4$ NaOH per 100 cc. milk) cc.
0	5·96	0·020	0·194	0·64
2	6·16	0·045	0·186	0·80
10	6·60	0·015	0·163	0·64

It will be observed that the residue and the dialysate obtained by the continuous dialysis method have both been analysed, whilst the composition of the dialysate has also been calculated from the difference between the analyses of the original milk and that of the residue. These calculated figures for the dialysate are considered more accurate than the determined values, which were subject to inaccuracies incurred in the bulking of numerous samples. Nevertheless, in the circumstances there is satisfactory agreement in most cases between the calculated and the determined values for the dialysate. In the case of the static dialysis the phosphorus distribution of the non-dialysable portion cannot be determined and therefore only the calculated composition from the difference between the determinations made on the dialysate and on the original milk is given.

As before, comment on these results is reserved for the general discussion.

DISCUSSION.

The phosphorus contents recorded in Tables III and IV are calculated as percentages of the non-fatty solids of the separated milk used, in order to facilitate comparison between the different milks.

Examination of the results both of the static dialysis and of the ultrafiltration, demonstrates that when separated milk powder "solution" is stored at 0° to 5° no significant change occurs in the phosphorus distribution, for after 2, 3, 4, 6 and 8 days the figures are materially the same; they have therefore been averaged. The figures so obtained show that the sera resulting from the two methods are similar in their organic and inorganic phosphorus contents. This is confirmed by the results obtained with the further series of milk powders (Table VI).

Table VI. *Comparison of the phosphorus distribution of different batches of spray-dried milk powder.*

Figures represent percentages.

		Exp. 4				Exp. 5			
		O.M.	S.D.	Ultrafiltrate		O.M.	S.D.	Ultrafiltrate	
Time of storage at 0° to 5° (hrs.) ...		—	48	0	48	—	48	0	48
Calculated on non-fatty solids	Inorg. P	0·65	0·34	0·34	0·33	0·64	0·34	0·32	0·34
	Org. P (by diff.)	0·35	0·08	0·06	0·07	0·35	0·10	0·09	0·09
	Total P	1·00	0·42	0·40	0·40	0·99	0·44	0·41	0·43
Calculated on total phosphorus of original milk	Inorg. P	65	34	34	33	65	34	32	34
	Org. P (by diff.)	35	8	6	7	35	10	9	9
	Total P	100	42	40	40	100	44	41	43

		Exp. 6				Exp. 7			
		O.M.	S.D.	Ultrafiltrate		O.M.	S.D.	Ultrafiltrate	
Time of storage at 0° to 5° (hrs.) ...		—	48	0	48	—	48	0	48
Calculated on non-fatty solids	Inorg. P	0·69	0·34	0·32	0·34	0·68	0·33	0·32	0·33
	Org. P (by diff.)	0·31	0·09	0·09	0·08	0·31	0·08	0·09	0·09
	Total P	1·00	0·43	0·41	0·42	0·99	0·41	0·41	0·42
Calculated on total phosphorus of original milk	Inorg. P	69	34	32	34	68	33	32	33
	Org. P (by diff.)	31	9	9	8	32	8	9	9
	Total P	100	43	41	42	100	41	41	42

O.M. = original milk. S.D. = static dialysate.

Examination of the figures obtained for the dialysate by static dialysis of freshly separated milk, Table IV, shows that no apparent change occurs on storing at 0° to 5° for 10 days, a result in agreement with the findings obtained using separated milk powder "solution." This is confirmed also by the results of the continuous dialysis, although the figures are subject to greater variation due to the more complicated technique.

The acid contents of the milk show very little change during the time of storage (Table V) and although variations in acidity do not necessarily indicate the presence or absence of proteolytic bacteria, it can be assumed from the results that little change of the type usually experienced in non-pasteurised milk had taken place.

These facts, together with the apparent constancy of the phosphorus results, were sufficient justification for averaging the various inorganic, organic and total phosphorus contents of the dialysate, Table IV.

On comparing these averaged results of the continuous dialysis with those of the static dialysis, it is evident that much more inorganic phosphorus is dialysed by the former method, while the amounts of dialysable organic phosphorus are identical in both cases. This is taken to indicate that the continuous method disturbs the salt equilibrium by dilution causing increased solution of inorganic phosphate, presumably calcium phosphate, but does not so affect the phosphorus organically combined.

When the static dialysis or ultrafiltration experiments of the "solution" of dried milk, Tables III and VI, are compared with the static dialysis experiment of liquid milk, Table IV, it is seen that much more phosphorus, both total and inorganic, occurs in the dialysate from the liquid milk. This observation appears to be in agreement with the findings of other workers; Magee and Harvey [1926] and Bell [1925] may be cited, who state that the action of heat on milk is to cause a reduction in soluble calcium and phosphorus probably by the production of colloidal calcium phosphate. This change might possibly be induced by the condensing and drying processes in the manufacture of powder. Obviously one cannot make any general statement as the original milks were all different, but it is to be remembered that in each case the experiment was carried out on samples of bulked milk.

Caution must be observed in commenting on the amount of the organic phosphorus (the difference between total and inorganic) but certainly it is remarkably constant in all instances even under the drastic treatment of continuous dialysis.

Comparison of the results here recorded with those of previous workers is practically impossible since no really adequate series of results is available. The results collected in Table I are abstracted from papers, the chief purport of which was not necessarily that of the distribution of phosphorus in milk, and therefore the figures are not at all complete. The figures reported herein (Tables VI and VII) show that 37–44 % of the phosphorus is in the dialysable form in separated milk powder "solution" and 46 % in liquid separated milk (Table VII), while 60 % becomes dialysable under the conditions of continuous dialysis.

The phosphorus analyses of sera obtained by other workers (Table I) fall between our values for ultrafiltrate or static dialysate and those for dialysate by the continuous method. This is what would be expected since certain of the

Table VII.

		Solution of spray-dried separated milk powder						Freshly separated liquid milk Experiment III		
		Experiment I			Experiment II			Average of three sets of results		
		Original milk %	Static dialysate %	Ultra-filtrate %	Original milk %	Static dialysate %	Ultra-filtrate %	Original milk %	Static dialysate %	Continuous dialysate %
	Total solids	9·85	—	—	9·32	—	—	8·80	—	—
	Fat	0·10	—	—	0·09	—	—	0·08	—	—
Calculated on non-fatty solids	Inorg. P	0·71	0·34	0·34	0·70	0·31	0·30	0·71	0·42	0·57
	Org. P (by diff.)	0·30	0·07	0·07	0·36	0·08	0·07	0·34	0·06	0·06
	Total P	1·01	0·41	0·41	1·06	0·39	0·37	1·05	0·48	0·63
Calculated on total phosphorus of original milk	Inorg. P	70·00	34·00	34·00	66·00	30·00	29·00	68·00	40·00	54·00
	Org. P (by diff.)	30·00	7·00	7·00	34·00	7·00	6·00	32·00	6·00	6·00
	Total P	100·00	41·00	41·00	100·00	37·00	35·00	100·00	46·00	60·00

methods adopted involve either direct or indirect dilution of the milk with consequent increase in the inorganic phosphorus of the serum.

It appears justifiable to average the results obtained from the six experiments using dried milk "solution" in view of the smallness of the differences observed. The phosphorus calculated on the total non-fatty solids is therefore:

	Total P %	Inorganic P %	Organic P %
Original milk	1·01	0·68	0·33
Serum	0·40	0·33	0·07

In other words, either by static dialysis or by ultrafiltration using collodion thimbles prepared as described, about 40 % of the total phosphorus, nearly 50 % of the inorganic phosphorus and a little more than 20 % of the organic phosphorus passes into the serum.

SUMMARY.

1. A continuous dialysis apparatus using collodion thimbles has been described which enables dialysis to be carried out at a low temperature (0° to 5°) and by means of which the dialysate can be concentrated to a convenient volume and the volume of distilled water used brought to a minimum. By applying a negative pressure to the outside of the dialysing sac, ultrafiltration is combined with dialysis.
2. Dialysis of milk by this means has been compared with that by a "static" dialysis method in which minimum dilution of the milk occurs; the dialysate so obtained has been compared with an ultrafiltrate. In all cases collodion thimbles were used. The comparisons have been made between the determined amounts of total and of inorganic phosphorus in the dialysis fractions and in the ultrafiltrates.
3. It has been shown that the serum obtained by "static" dialysis of a solution of spray-dried separated milk powder has approximately the same total and inorganic phosphorus content as has that of an ultrafiltrate from the same solution.
4. Continuous dialysis compared in a similar manner with static dialysis using fresh separated milk shows that the former method causes much more phosphorus to be dialysable and that this increase is confined entirely to the inorganic phosphorus, the dialysable organic phosphorus being the same in both cases.
5. The results recorded show a certain constancy in the phosphorus distribution of the particular brand of spray-dried separated milk used. These results when compared with similar ones using bulked liquid separated milk support the contention of other workers that heat treatment reduces the inorganic phosphorus of the serum obtained by dialysis or by ultrafiltration.
6. Sufficient evidence has not yet been obtained to make a definite statement regarding the distribution of phosphorus in milk and spray process powder made therefrom, but there are indications that the heating, or it may be the desiccation, causes slight alterations in the relative amount of phosphorus dialysable.

Our thanks are due to Messrs J. Lyons and Co., Ltd., in whose laboratories this work was conducted, for permission to publish; also to Miss D. F. Filmer and to Mr R. F. Smith who have carried out a large proportion of the experimental work, and to Mr E. B. Hughes for valuable criticism.

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XCV. THE DETERMINATION OF REDUCING SUGARS BY TITRATION OF FERRICYANIDE.

By SYDNEY WILLIAM COLE.

From the Biochemical Laboratory, Cambridge.

(*Received April 7th, 1933.*)

The use of ferricyanide for the quantitative estimation of sugar was introduced by Hagedorn and Jensen [1923]. Their method has been extended to other sugars by Hanes [1929] and Hulme and Narain [1931]. These methods involve a preliminary heating for 15 minutes and a subsequent back titration.

The fact that ferricyanide is only slightly coloured would seem to make it valueless for a direct titration method. In spite of this Hawkins and Van Slyke [1929] have introduced a timing method for the estimation of glucose in blood and urine, based on the time required for the decoloration of alkaline ferricyanide when immersed in a boiling water-bath. They state that a little practice is necessary to train the eye in judging the moment at which the yellow colour vanishes. I personally find it extremely difficult to know when to stop the watch but am more concerned with the fact that they make no mention of the variation due to alterations in barometric pressure. I discarded a timing method based on the decoloration of an alkaline copper solution when immersed in boiling water owing to this difficulty.

Lane and Eynon [1923] introduced methylene blue as an internal indicator for the detection of the end-point when titrating Fehling's solution with reducing sugars. I have used this method with good success in class work for some years. It is admirable for the estimation of pure sugar solutions, but my students find it difficult to detect the end-point when applying it to urine. The colloidal substances in urine inhibit the aggregation of the cuprous oxide to the red form, so that the end-point is seen by a slight decrease in total colour rather than in a disappearance of the blue tint. It is particularly difficult to see this by artificial light.

I find that the addition of methylene blue to an alkaline solution of ferricyanide enables one to estimate reducing sugars by direct titration very rapidly and accurately. The indicator is not reduced until the whole of the ferricyanide has been reduced and the end-point, being a change from a blue or violet solution to one that is colourless, is unmistakeable.

Solutions.

(1) Potassium ferricyanide, 1 %. This should be stored in a dark bottle and kept in a dark cupboard when not in use. I have not determined its stability, but have been unable to detect any change over a period of 6 weeks.

(2) Sodium hydroxide, 2·5 *N*. This is best prepared by decanting or filtering the clear fluid from some 45 % NaOH that has stood for a few days and diluting down to about 11 %. 10 cc. are titrated with *N* HCl against methyl red. The bulk is then diluted so that 10 cc. of the NaOH require 25 cc. of the acid.

(3) Methylene blue, 1 % in water. This is conveniently stored in a bottle fitted with a rubber cork, carrying a dropping pipette with a rubber teat.

Apparatus. 100 cc. flasks with rather narrow necks.

Graduated 2 and 5 cc. pipettes. I prefer these to burettes for accurate delivery into boiling fluids. By holding the pipette nearly horizontally it will be found that deliveries of about 0·02 cc. can be made, the condensing steam washing the drop off the end of the pipette.

Broken porcelain. I find it necessary to add a pinch of clean, dry porcelain chips the size of small glass beads to ensure smooth boiling.

Rough titration. To 20 cc. of the ferricyanide and 5 cc. of the NaOH in a 100 cc. flask, add a pinch of broken porcelain. Heat to boiling on a wire gauze over a Bunsen flame. Add the sugar solution slowly until the yellow colour has appreciably decreased. Then add a small drop of methylene blue. Continue to add the sugar until the fluid is decolorised, allowing a few seconds between each addition. The exact equivalents are given below, but as a rough guide it can be stated that about 1 cc. of a 2 % or 2 cc. of a 1 % solution of glucose are necessary.

Final titration. The standard conditions are as follows. To 20 cc. of the ferricyanide and 5 cc. of the NaOH are added a pinch of broken porcelain and one small drop of methylene blue. To the cold mixture is added all but about 0·2 cc. of the volume of sugar solution judged to be necessary from the preliminary trial. The mixture is brought to the boil in about $1\frac{3}{4}$ min. The flame is then lowered a little, so that only gentle boiling is obtained. After 1 min. the remaining sugar is added, a drop at a time, at 10 or 15 sec. intervals, until the end-point is reached. The total boiling time should be over 2 min. and must not exceed 3 min.

If less than 2 cc. are required, I generally dilute so that between 3 and 5 cc. are necessary. If more than 10 cc. are necessary I prefer to use a burette with a pinch cock.

Calculation of results. The factors obtained below were determined by the use of a variety of pure sugars, the exact strengths of which were obtained polarimetrically. I find that the amount of sugar required to reduce the ferricyanide increases slightly with an increase in the volume of fluid added. This is due to the decrease in the alkalinity.

If x be the volume of sugar solution added (in cc.), then the amount of the sugar required to reduce the ferricyanide is: glucose, $20{\cdot}12 + 0{\cdot}035x$ mg; maltose anhydride, $26{\cdot}8 + 0{\cdot}06x$ mg.; lactose anhydride, $23{\cdot}6 + 0{\cdot}1\,x$ mg.

Thus if 4 cc. of a glucose solution are required, then 4 cc. contain $20{\cdot}12 + 0{\cdot}14$ mg. and 100 cc. contain 0·506 g.

Or generally if x cc. are required, then g. per 100 cc. is $2{\cdot}012/x + 0{\cdot}035$.

This relation is expressed graphically by the curves in Fig. 1.

Estimation of sucrose. The sucrose was inverted by adding 10 cc. of 0·1 N HCl to 25 cc. of the solution, boiling gently for 10 mins. in a Kjeldahl flask with a long neck, cooling, adding 10 cc. of 0·1 N NaOH and making up to 50 or 100 cc. The factor for sucrose originally present is $19{\cdot}2 + 0{\cdot}065x$ mg.

The estimation of mixtures of maltose and glucose. I find that complete hydrolysis of maltose without loss of glucose is effected by boiling 25 cc. of the solution with 20 cc. of N HCl in a Kjeldahl flask with a long neck for 30 mins., 10 to 15 cc. of water being added after 15 mins. to prevent undue concentration. The mixture is cooled, accurately neutralised to methyl red with $2{\cdot}5N$ NaOH and made up to 100 cc., *i.e.* 1 in 4 dilution.

Two estimations of the reducing power (in terms of g. of glucose per 100 cc.) are made; B (before hydrolysis) and A (after hydrolysis).

The following example illustrates the method of calculation.

The solution used was prepared by mixing equal volumes of a 2·1 % solution of glucose and a 3·72 % solution of maltose anhydride.

B (using a 1 in 4 dilution) required 3·30 cc.

A (using a 1 in 4 dilution) required 2·68 cc.

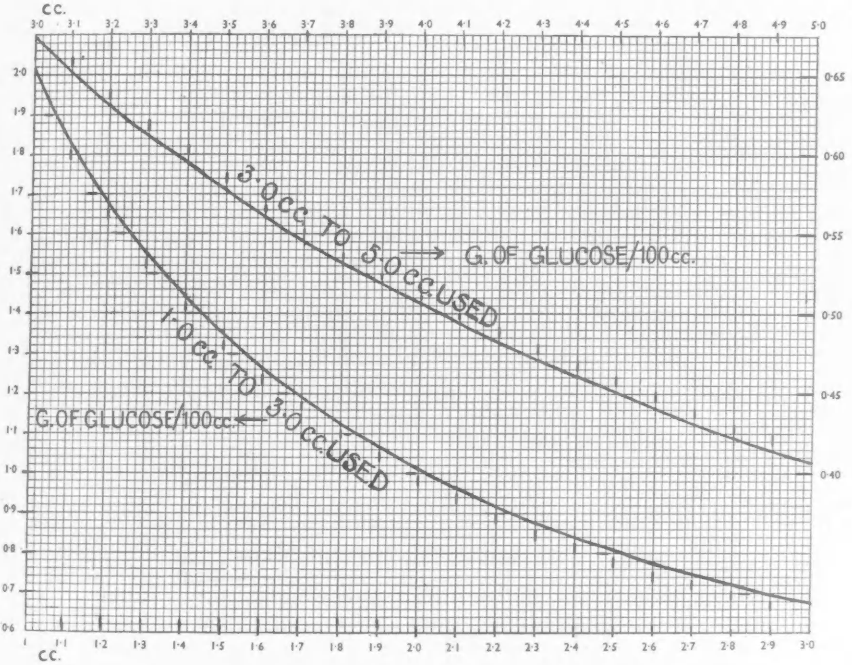

Fig. 1. Curve for reading g. of glucose/100 cc. from volume used.

The reducing power of maltose is less than that of glucose in the ratio of $(20{\cdot}12 + 0{\cdot}035 \times 3{\cdot}3)$ to $(26{\cdot}8 + 0{\cdot}06 \times 3{\cdot}3) = 0{\cdot}749$ to 1. That is 1 g. of maltose behaves like 0·749 g. of glucose when 3·3 cc. of sugar solution are used.

If there be G g. of glucose and M g. of maltose in 100 cc. of the original solution, then

$$G + 0{\cdot}749\, M = \frac{(20{\cdot}12 + 0{\cdot}035 \times 3{\cdot}3) \times 4}{3{\cdot}3 \times 10} = 2{\cdot}452 \qquad \text{......(B).}$$

By boiling with acid 1 g. of maltose is converted to 1·053 g. of glucose. So in the second estimation

$$G + 1{\cdot}053\, M = \frac{(20{\cdot}12 + 0{\cdot}035 \times 2{\cdot}68) \times 4}{2{\cdot}68 \times 10} = 3{\cdot}016 \qquad \text{......(A).}$$

Subtracting B from A, $0{\cdot}304\, M = 0{\cdot}564$ and $M = 1{\cdot}865$. Substituting this value for M in A, we get $G = 1{\cdot}052$. The analytical results agree almost exactly with theory, G 1·05 and M 1·86.

The estimation of lactose in milk. To 25 cc. of water in a 100 cc. flask add 5 cc. of the milk. Measure 5 cc. of 5 % "colloidal iron" in a pipette. Add this to the diluted milk, mixing by gentle agitation during the addition. Seal the flask by the thumb and give a shake. Filter through a dry paper and estimate as described above. Most specimens of cow's milk require between 3 and 4 cc. of this 1 in 7 dilution.

Calculation. If x cc. of the filtrate are required the lactose anhydride in g./100 cc. is

$$\frac{(23{\cdot}6+0{\cdot}1x)\times 7}{x\times 10}=\frac{16{\cdot}52}{x}+0{\cdot}07.$$

The estimation of glucose in urine. Urates reduce alkaline ferricyanide at room temperature. For clinical work I find that almost exact results are obtained by treating the urine with 4 % of a good charcoal. The best for this purpose seems to be "actibon," which can be obtained from Baird and Tatlock, 14, Cross Street, London. Only a minute trace of urate or creatinine can be found in the filtrate, and the amount of glucose adsorbed is so small that results are correct to within 2 %. The end-point is as sharp as in the case of pure sugar solutions, and thus differs from all the direct titration methods involving copper solutions that I have tried. The method is as follows. To 1 g. of "actibon" charcoal in a small beaker add 25 cc. of the urine. Stir well and filter after about a minute. Estimate the sugar in the filtrate as described above. If the glucose is more than 1 % the operator will probably prefer to dilute so that between 3 and 5 cc. are necessary. But it must be noted that it is not advisable to dilute the urine before treatment with charcoal, as there is a chance of a loss of glucose, which is adsorbed from pure solution and from very dilute urines, but only very slightly from normal urine [Cole, 1913].

SUMMARY.

A method is described for the estimation of reducing sugars by the direct titration of boiling alkaline ferricyanide, containing a drop of methylene blue as an internal indicator.

Equivalents are given for glucose, maltose and lactose.

A method for the estimation of sucrose is described.

Details are given for the estimation of lactose in milk and of glucose in urine.

A method is described for the estimation of maltose and glucose in a mixture of these sugars.

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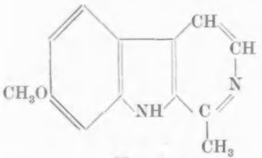
XCVI. THE CHEMOTHERAPY OF DERIVATIVES OF HARMINE AND HARMALINE. I.

By CHARLES EDWARD COULTHARD,
HYMAN HENRY LIONEL LEVENE
AND FRANK LEE PYMAN.

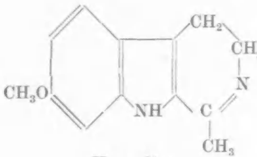
From the Research Department, Boots Pure Drug Co., Ltd., Nottingham.

(*Received April 26th, 1933.*)

THIS investigation had its origin in Gunn and Marshall's [1920] discovery that harmine and harmaline had some therapeutic value in malaria. Further clinical trials of these compounds, however, failed to establish their practical worth as antimalarial agents. Since harmine and harmaline are readily accessible in quantity by extraction from *Peganum harmala*, and their chemical constitution, determined by Perkin and Robinson [1919, 2],



Harmine



Harmaline

has features in common with those of known antimalarial agents such as quinine and plasmoquin, we thought it of interest to prepare a number of derivatives of these alkaloids in order that they might be tested for antimalarial action. Now, Morgenroth and his collaborators have shown that the bactericidal value of a homologous series of *O*-alkylhydrocupreines increases to a peak value and then falls again. This fact suggested to us that perhaps replacement of the methoxy-group of harmine or harmaline by higher alkyloxy-groups might yield substances of greater antimalarial action. Suitable methods for the demethylation of these alkaloids and subsequent *O*-alkylation of the resulting phenolic compounds, harmol and harmalol, were worked out, and all the members of the homologous normal series from *O*-ethylharmol to *O*-*n*-dodecylharmol (except undecylharmol), several members of the *iso*-series and also *O*-allyl- and *O*-benzylharmol were prepared. A smaller number of the corresponding derivatives of harmalol were also made.

Through the courtesy of the Chemotherapy Committee of the Medical Research Council, two members of the series, namely, *O*-*n*-butylharmol and *O*-*n*-heptylharmol, as well as harmol, harmalol and *O*-β-diethylaminoethylharmol (to which reference will be made later) were tested for activity against bird-malaria under the direction of Prof. Keilin, of the Molteno Institute, Cambridge, but were found to be inactive.

The possibility that some members of these series might have other chemotherapeutic uses was then examined, and it was found that both bactericidal and amoebicidal activity increased, on ascending the homologous series, up to a point and then started to fall. The peak of amoebicidal activity was reached at *O-n*-nonylharmol, but the salts of this and other high members of the series were very sparingly soluble in water, and consequently a further series of compounds was prepared with the hope of obtaining more readily soluble compounds. In the first place, the effect of an ω-hydroxy-group was tested by the preparation of *O*-ω-hydroxy-*n*-decylharmol, but the salts of this were also sparingly soluble. A series of *O*-ω-dialkylamino-*n*-alkylharmols was then prepared, and these were found to yield dihydrochlorides readily soluble in water. An account of the investigation of the bactericidal properties of these and the simpler alkylharmols follows. Many of the ω-dialkylamino-*n*-alkylharmols proved to have a very considerable amoebicidal action, when tested *in vitro* by the method of Laidlaw, Dobell and Bishop [1928], and the results obtained in this connection will be reported later. Three members of the series, *O-n*-butylharmol, *O*-ω-diethylamino-*n*-heptylharmol, and *O*-ω-diethylamino-*n*-decylharmol were tested for trypanocidal action by Mr W. A. Broom, of Boots Pharmacological Department, but were found to be inactive in doses of approximately 50 % of the lethal dose.

It may be noted that the pharmacology of the *O*-ethyl, *n*-propyl, *n*-amyl, and *n*-nonyl ethers of harmol has been studied by Gunn and Heathcote [1931], Elphick and Gunn [1932], and Gunn and MacKeith [1932].

BACTERICIDAL PROPERTIES.

The methods to be adopted in the examination of new compounds for bactericidal properties call for very careful consideration. It is obviously impracticable to investigate the activity of all of a series of compounds from every point of view, and in the case of homologous series of compounds we have adopted the procedure of exploring the series for "peak" activity by some particular test and later testing out the "peak" compound more thoroughly. In the testing of these compounds for bactericidal efficiency, we have used the Rideal-Walker technique for the preliminary survey, and since it is well known that the "peak" bactericidal activity of a homologous series of compounds differs with the test organism, we have in some cases supplemented our results on *Bacillus typhosus* with others obtained using *Staphylococcus aureus*. These two organisms may, we believe, be taken as representative of two important groups of bacteria, that of the organisms allied to *B. coli*, and that of the Gram positive cocci. Both organisms will grow in the Rideal-Walker broth and may be used for the Rideal-Walker test.

Table I shows the results of our Rideal-Walker (phenol coefficient) tests on the hydrochlorides of these compounds.

It will be observed that in the *n*-alkyl series the peak of bactericidal efficiency is reached at butyl for *B. typhosus*, and at amyl for *S. aureus*, and that where the *n*-members are highly active, corresponding *iso*-members are less so. In the ω-diethylaminoalkyl series the peak for *B. typhosus* is reached at diethylaminononylharmol, and in the dialkylaminodecyl series at the diethylamino-member.

O-n-Butylharmol hydrochloride, with a coefficient against *B. typhosus* of 350 and against *S. aureus* of 150, is evidently a germicide of great potency and additional work has been carried out to study its action more fully. The inhibitory effect and the lethal effect in the presence of blood and serum were tested

Table I.

Compound	R.W. coefficients	
	B. typhosus	*S. aureus*
Harmol	<1	—
Harmine	<1	—
O-Ethylharmol	25	—
O-*n*-Propylharmol	225	75
O-*n*-Butylharmol*	350 to 400	150
O-*n*-Amylharmol	350	250 to 300
O-*n*-Hexylharmol	50	45 to 50
O-*n*-Heptylharmol	30 to 35	45 to 50
O-*n*-Octylharmol	15	35 to 40
O-*n*-Nonylharmol	10 to 15	15
O-*n*-Decylharmol	10	—
O-*n*-Dodecylharmol	5	—
O-*iso*-Propylharmol	75	—
O-*iso*-Amylharmol	150 to 200	—
O-*sec*.-Octylharmol	About 20	—
O-*iso*-Decylharmol	10	—
O-Allylharmol	110	—
O-Benzylharmol	250	—
O-ω-Hydroxydecylharmol	10 to 15	—
O-β-Diethylaminoethylharmol	5	—
O-ω-Diethylaminoheptylharmol	23	<12
O-ω-Diethylaminooctylharmol	110	†About 100
O-ω-Diethylaminononylharmol	220	†About 200
O-ω-Diethylaminodecylharmol	200	†250 to 300
O-ω-Diethylaminoundecylharmol	160	†About 200
O-ω-Dimethylaminodecylharmol	125	—
O-ω-Dibutylaminodecylharmol	150	—
O-ω-Piperidyldecylharmol	55	—
Harmaline	<1	—
Harmalol	<1	—
O-Ethylharmalol	2–5	—
O-*n*-Butylharmalol	About 100	—

* *O*-*n*-Butylharmol methochloride had a R.W. coefficient of 50 to *B. typhosus*.

† In our hands the *S. aureus* coefficients of these four compounds differ widely from test to test. The results quoted were obtained in tests put on at one time.

using cocci and *Bacillus coli*. In all cases where a test against cocci is described the test culture was prepared by mixing, immediately before use, 24-hour broth cultures of all the available strains of *S. aureus* and of streptococci. The following results are of interest.

(1) *Inhibition tests.*

A. *In broth* (prepared by tryptic digestion of ox-heart).

B. coli was inhibited by 1 in 10,000 to 1 in 25,000.

Cocci were inhibited by 1 in 50,000 to 1 in 100,000.

Aspergillus (*Sterigmatocystis*) *niger* was inhibited by 1 in 25,000 to 1 in 75,000.

Using phenol under the same conditions the figures were, for *B. coli*, 1 in 500; for cocci, 1 in 250; and for *A. niger*, 1 in 1200.

B. *In broth with glucose 1* $^0/_0$ *and ox-serum 20* $^0/_0$.

B. coli was inhibited by 1 in 5000 to 1 in 10,000.

Cocci were inhibited by 1 in 2500 to 1 in 7500.

Using phenol under the same conditions the figures were, for *B. coli*, 1 in 400; and for cocci, 1 in 200.

C. *Using the serum-agar cup test of Himebaugh* [Allen and Wright, 1931].

With *S. aureus*, and allowing 24 hours' contact, 1 in 500 *O*-*n*-butylharmol hydrochloride gave a "halo" of similar extent to that produced by 5 % phenol.

(2) *Tests to indicate the lethal efficiency in blood or in serum.*

A. *Germicidal effect in the presence of serum.*

(0·5 cc. of equal parts of broth culture of cocci and ox-serum added to 5 cc. of the germicide dilution in equal parts of serum and saline.)

A dilution of 1 in 500 killed in about 4 hours.

1 in 1000 did not kill in 48 hours.

Corresponding dilutions of phenol:

1 in 50 killed in under 4 hours.

1 in 100 killed in 24 hours, not in 8.

B. *Germicidal effect in the presence of blood.*

(1 cc. of equal parts of culture and citrated blood added to 5 cc. of germicide solution.)

Using cocci as previously described complete sterilisation was obtained in less than 5 minutes with dilutions of 1 in 1000 to 1 in 2500.

Of a number of germicides tested in this way, none was as efficient as *O-n*-butylharmo hydrochloride. To effect sterilisation in 5 minutes the following concentrations were necessary.

O-n-Butylharmol hydrochloride	1–1000 to 1–2500
Brilliant green	1–200
Mercuric iodide	1–200
Silver nitrate	1–200
Neutral acriflavine	1–100
Lysol	1–50
Phenol	1–25

C. *Action on phagocytosis.*

Fleming [1931], has shown that the addition of most germicides to living blood reduces the efficiency of the antibacterial action of the leucocytes long before the germicide is in sufficient concentration to exert any bacteriostatic action. We have carried out a number of "slide cell" tests [Wright, Colebrook and Storer, 1923], in which dilutions of germicides in Ringer's solution were added to *Streptococcus faecalis* in fresh citrated rabbit blood and, slide cells being prepared and incubated, the developing colonies read off after incubation.

Our tests indicate that *O-n*-butylharmol hydrochloride also is more toxic to leucocytes than to bacteria. In a typical result, a control, in which the leucocytes were inactivated by strong saline, showed 35 colonies, another control to which Ringer's solution only was added showed 1 colony; the following figures indicate the colonies developed in the presence of different concentrations of *O-n*-butylharmol hydrochloride.

1–1750	0 colonies
1–3500	30 colonies
1–7000	0 colonies
1–14,000	0 colonies

From a consideration of this bacteriological work it appears that this compound does not possess the power of acting in extreme dilution, whether blood-serum be present or not, so characteristic of acriflavine.

On the other hand, none of the germicides we have tested approaches *O-n*-butylharmol hydrochloride in the efficiency and rapidity of its action against cocci in the presence of blood, and it appears possible that the compound might have some application in the disinfection of discharges containing blood where the action of phagocytes was unimportant.

Preparation of harmol and harmalol.

Harmol and harmalol have been prepared by the demethylation of harmine and harmaline respectively using hydrochloric acid under pressure [Fischer and Täuber, 1885; Fischer, 1889], and in the former case by means of boiling hydriodic acid also [Perkin and Robinson, 1919, 2]. They can be prepared more conveniently by boiling with diluted sulphuric acid [Boots Pure Drug Co., Ltd., Pyman and Levene, 1932, 1]. A nearly theoretical yield of harmol can be obtained by boiling harmine with sulphuric acid of 35 to 60 % strength, the time required for the hydrolysis decreasing with the strength of acid from 100 to about 2 hours. When stronger sulphuric acid is employed, the product is contaminated with harmolsulphonic acid.

Harmol. Harmine (120 g.), is boiled with a mixture of sulphuric acid (1200 g.), and water (800 cc.) until a test portion is completely soluble in aqueous sodium hydroxide (about 2 hours). On cooling harmol sulphate crystallises. It is collected, suspended in hot water and mixed with sodium carbonate, yielding pure harmol (109 g., *i.e.* nearly theoretical yield, M.P. 320° decomp.).

The *hydrochloride* crystallises from water in minute colourless needles with $\frac{1}{2}H_2O$, which do not melt below 350°. It is soluble in 23 parts of cold water, giving a colourless fluorescent neutral solution. (Found, in air-dried salt: loss at 100°, 3·9; N, 11·5; Cl, 14·7 %. $C_{12}H_{10}ON_2$, HCl, $\frac{1}{2}H_2O$ requires H_2O, 3·7; N, 11·5; Cl, 14·6 %.) The *lactate* crystallises from moist acetone in colourless needles, M.P. 207–208° (corr.). It is soluble in 4 parts of cold water. (Found: C, 62·7; H, 5·7 %. $C_{12}H_{10}ON_2$, $C_3H_6O_3$ requires C, 62·5; H, 5·6 %.)

Harmolsulphonic acid is obtained together with harmol by heating harmine with sulphuric acid of more than 70 % strength at 150°, the proportion of sulphonic acid increasing rapidly with increasing concentration of acid. The solution is heated until a sample is completely soluble in caustic soda. The concentration of sulphuric acid is then reduced to 50 % by adding water, and on cooling harmol sulphate crystallises out at once with a little harmolsulphonic acid, the bulk of the sulphonic acid coming down on standing. The crude acid is freed from harmol by adding sodium acetate and dilute acetic acid and is then recrystallised from much boiling water, when it separates in greyish needles with $4H_2O$. It does not melt below 300°, is sparingly soluble in water, and its sodium and ammonium salts are also sparingly soluble in water. (Found, in air-dried substance: loss at 120°, 20·7; S, 9·1 %. $C_{12}H_{10}O_4N_2S$, $4H_2O$ requires H_2O, 20·6; S, 9·2 %.)

Harmalol is readily made by boiling harmaline under reflux with 20–55 % sulphuric acid, the operation requiring approximately 100 and 4 hours in the extreme cases. With stronger sulphuric acid, the product is contaminated with harmalolsulphonic acid. Harmalol is most conveniently made by gently boiling harmaline with 20 parts by weight of 50 % sulphuric acid, until a test sample is completely soluble in aqueous sodium hydroxide (6 to 8 hours). On cooling, harmalol sulphate crystallises. It is collected and mixed with hot water and ammonia, when the pure base is obtained in nearly theoretical yield, and has the properties described by previous authors. The *lactate* crystallises from alcohol in bright yellow microscopic leaflets containing $1H_2O$. M.P. (air-dried) 116–120° (corr.); (anhydrous) 174–176° (corr.). It is soluble in an equal weight of water, and is readily soluble in alcohol. (Found: loss at 100°, 5·9; C, 58·4; H, 6·6; N, 9·3 %. $C_{12}H_{12}ON_2$, $C_3H_6O_3$,H_2O requires H_2O, 5·9; C, 58·5; H, 6·5; N, 9·1 %.)

Harmalolsulphonic acid is obtained together with harmalol by heating harmaline with sulphuric acid exceeding 60 % in strength, at 150–160°. It can be isolated by repeatedly extracting the mixture of crude harmalol sulphate and sulphonic acid with hot dilute aqueous ammonia, and acidifying the solution. It is thus obtained as a crystalline yellow powder, containing $1H_2O$. It does not melt below 300°, and is sparingly soluble in water. (Found, in air-dried substance: loss at 100°, 6·5; S, 10·8 %. $C_{12}H_{12}O_4N_2S$, H_2O requires H_2O, 6·1; S, 10·8 %.)

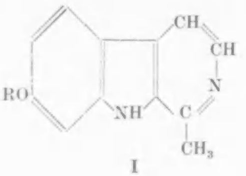
Alkylation of harmol and harmalol.

Harmol and harmalol present two points of attack to alkylating agents and can give *O*-alkyl, *N*-alkyl, and *ON*-dialkyl derivatives. In the case of harmine, the formulae of these substances are given on p. 732.

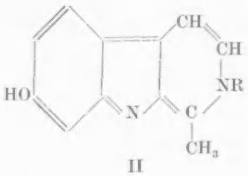
Formulae II and III arise by a molecular rearrangement which has been explained by Perkin and Robinson [1919, 1].

The results of alkylation depend upon the conditions employed. On alkylating harmol in alcoholic solution in the presence of sodium carbonate with butyl *p*-toluenesulphonate (1 mol.) for example much harmol is recovered unchanged, and *ON*-dibutylharmol is formed, identical with the product obtained by heating the dry sodium salt of harmol with butyl *p*-toluenesulphonate (2 mols.) in the absence of a solvent.

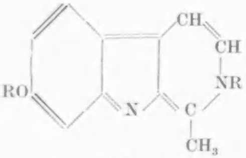
If however harmol is heated under reflux in alcoholic solution with sodium hydroxide (1 mol.) and an alkyl or aralkyl salt (1 mol.) the main product of the reaction is an *O*-alkyl or aralkyl ether of harmol [Boots Pure Drug Co., Ltd., Pyman and Levene, 1932, 2].



I



II



III

The alkyl salts usually employed were bromides, but other salts were used in some cases, *e.g.* allyl and octyl iodides, benzyl chloride, butyl *p*-toluenesulphonate. The product of the reaction was worked up by distilling off the alcohol, and mixing the residue with dilute aqueous sodium hydroxide to remove unchanged harmol and the *N*-alkylharmol. The insoluble residue—a solid or with the higher aliphatic derivatives an oil which rapidly crystallised—was yellow in colour owing to the presence of a small proportion of the *ON*-dialkyl derivative. Purification was best effected by recrystallising the hydrochloride from alcoholic hydrochloric acid, with the lower members of the series, or from alcohol-acetone with the higher members. The bases were best obtained pure by adding excess ammonia to a hot dilute solution of the hydrochloride in dilute alcohol.

The *O*-alkylharmols crystallise from dilute alcohol in the form of colourless needles which are anhydrous. They are almost insoluble in water, but readily soluble in alcohol and chloroform, and slightly soluble in ether and benzene. They form colourless hydrochlorides crystallising in needles, some with and others without water of crystallisation and are moderately or slightly soluble in water, the solutions being colourless, fluorescent and neutral to litmus. The behaviour of the hydrochlorides of the members of the *n*-series from butyl upwards is peculiar. In the case of the butyl ether and the near higher homologues, a hot strong aqueous solution gelatinises on cooling. From the *n*-octyl ether upwards, the salts are sparingly soluble even in hot water but become gelatinous on contact with water. The amyl derivative and its higher homologues gradually hydrolyse in solution, depositing crystals of the base. The lactates are far more soluble than the hydrochlorides, do not gelatinise so readily and—possibly because of their increased concentration—show little or no hydrolysis even on prolonged standing.

N-alkylated derivatives, *e.g.* metho- or buto-chlorides do not gelatinise in the same way as the hydrochlorides and are also moderately or sparingly soluble in water. They can be made by the action of an alkyl halide on the alkyl ether in benzene solution.

Harmalol was alkylated in exactly the same way as harmol. The *O*-alkyl ethers of harmalol form yellow salts, which are considerably more soluble than the corresponding harmol derivatives, their solutions being yellow, highly fluorescent and neutral to litmus.

O-*Ethylharmol.* Harmol (8 g.), ethyl bromide (4·5 g.), aqueous 5*N* sodium hydroxide (8·1 cc.), and 99 % alcohol (200 cc.) were heated under reflux for 2 hours. After distilling off the alcohol the residue was washed with dilute aqueous sodium hydroxide to remove phenolic bases. The washings were acidified with acetic acid and then basified with ammonia, giving 2·6 g. of a somewhat tarry precipitate, mainly harmol. The residue insoluble in aqueous sodium hydroxide was a powdery solid (6·1 g.) pale yellow in colour due to the presence of some *ON*-diethylharmol. It was dissolved in hot alcohol (18 cc.), and concentrated hydrochloric acid (9 cc.) was added; on cooling, the crude hydrochloride (6·6 g.) crystallised.

After recrystallisation from very dilute hydrochloric acid, using charcoal, the pure *hydrochloride* was obtained as a mass of colourless, anhydrous needles, M.P. 313° (corr.) after sintering from 306°. It is soluble in 12 parts of water at 80°, or 18 parts at 24°. (Found: Cl, 13·5 %, $C_{14}H_{14}ON_2$, HCl requires Cl, 13·5 %.)

The *free base* is obtained in long, anhydrous needles, M.P. 193° (corr.) on adding ammonia to a hot dilute solution of the hydrochloride in aqueous alcohol. (Found: C, 74·1; H, 6·3 %. $C_{14}H_{14}ON_2$ requires C, 74·3; H, 6·2 %.) The *lactate* crystallises from moist acetone in anhydrous needles, M.P. 174–175° (corr.), which are soluble in 3 parts of cold water. (Found: C, 64·3; H, 6·5 %. $C_{14}H_{14}ON_2$, $C_3H_6O_3$ requires C, 64·5; H, 6·3 %.)

The following homologues of *O*-ethylharmol were prepared similarly.

O-n-*Propylharmol*, thick needles, M.P. 203–204° (corr.). (Found: C, 74·6; H, 6·8 %. $C_{15}H_{16}ON_2$ requires C, 75·0; H, 6·7 %.) The *hydrochloride*, minute needles, M.P. 259–261° (corr.) soluble in 48 parts of water at 20°. (Found, in air-dried salt: loss at 100°, 3·2 %. $C_{15}H_{16}ON_2$, HCl, $\frac{1}{2}H_2O$ requires H_2O, 3·2 %. Found, in dried salt: Cl, 12·7 %. $C_{15}H_{16}ON_2$, HCl requires Cl, 12·8 %.) The *lactate*, long anhydrous needles, M.P. 164–166° (corr.), soluble in 2 parts of cold water. (Found: C, 65·5; H, 6·8 %. $C_{15}H_{16}ON_2$, $C_3H_6O_3$ requires C, 65·4; H, 6·7 %.)

O-iso*Propylharmol*, feathery needles, M.P. 181° (corr.). (Found: C, 75·2; H, 6·8 %. $C_{15}H_{16}ON_2$ requires C, 75·0; H, 6·7 %.) The *hydrochloride*, fine needles, M.P. 277–278° (corr.) after drying at 100°, soluble in 5 parts of water at 75° and 60 parts at 20°. (Found, in air-dried salt: loss at 100°, 6·1 %. $C_{15}H_{16}ON_2$, HCl, H_2O requires H_2O, 6·1 %. Found, in dried salt: Cl, 12·9 %. $C_{15}H_{16}ON_2$, HCl requires Cl, 12·8 %.)

O-*Allylharmol*, needles, M.P. 166–167° (corr.). (Found: C, 75·5; H, 6·0 %. $C_{15}H_{14}ON_2$ requires C, 75·6; H, 5·9 %.) The *hydrochloride*, small needles, M.P. 233° (corr.) when air-dried and M.P. 221–222° (corr.) after drying at 110°, soluble in 10 parts of cold water. (Found, in air-dried salt: loss at 110°, 6·2 %. $C_{15}H_{14}ON_2$, HCl, H_2O requires H_2O, 6·1 %.)

O-n-*Butylharmol*, thick rhombs, M.P. 220–221° (corr.). (Found: C, 75·3; H, 7·2 %. $C_{16}H_{18}ON_2$ requires C, 75·6; H, 7·1 %.) The *hydrochloride*, rhombs, M.P. 232–234° (corr.), is soluble in 25 parts of hot water giving a solution which sets to a firm gel on cooling. At 15° a 0·8 % solution can be poured. (Found: Cl, 12·3 %. $C_{16}H_{18}ON_2$, HCl requires Cl, 12·3 %.) The *lactate*, slender anhydrous needles, M.P. 160–163° (corr.) is soluble in 20 parts of cold water. The *methiodide*, obtained by heating the base with methyl iodide in benzene as a cream-coloured crystalline precipitate, M.P. 247° (corr.), is sparingly soluble in water, and gives, when boiled with an aqueous suspension of silver chloride, the *methochloride*, clusters of needles, M.P. 279° (corr.). (Found: Cl, 11·8 %. $C_{17}H_{21}ON_2Cl$ requires Cl, 11·7 %.) The methochloride is soluble in 10 parts of water at 70°, and this solution does not gelatinise but deposits crystals on cooling, the solubility of the salt at 31° being only 0·8 %. It yields with ammonia the corresponding base, *N*-methyl-*O*-*n*-butylharmol, which crystallises from alcohol in glistening dark green needles, M.P. 144° (decomp.).

ON-*Di*-n-*butylharmol*, prepared by heating harmol with butyl *p*-toluenesulphonate in the presence of sodium carbonate or by heating the sodium salt of harmol with butyl *p*-toluenesulphonate (2 mols.) crystallised from alcohol in yellow needles, M.P. 113° (corr.). (Found: C, 69·1; H, 8·4 %. $C_{20}H_{26}ON_2$, $2H_2O$ requires C, 69·3; H, 8·6 %.) Its *hydrochloride* or O-n-*butyl-*

harmol n-*butochloride* crystallised from water in colourless needles, containing $2H_2O$ lost at 100°. M.P. 68° (corr.). (Found: Cl, 9·25: loss, 9·44 %. $C_{20}H_{27}ON_2Cl, 2H_2O$ requires Cl, 9·30: loss, 9·35 %. The anhydrous salt melts at 185–186° (corr.).

O-n-*Amylharmol*, needles, M.P. 204–206° (corr.). (Found: C, 75·9; H, 7·5 %. $C_{17}H_{20}ON_2$ requires C, 76·1; H, 7·5 %.) The *hydrochloride*, long silky needles, M.P. 192–194° (corr.) after drying at 50°, is soluble in 10 parts of hot water, the solution gelatinising on cooling, sparingly soluble in cold water. (Found, in air-dried salt: loss at 50°, 5·2 %. $C_{17}H_{20}ON_2$, HCl, H_2O requires H_2O, 5·5 %. Found, in dried salt: Cl, 11·4 %. $C_{17}H_{20}ON_2$, HCl requires Cl, 11·7 %.) The *lactate*, colourless needles, M.P. 130–132° (corr.), sparingly soluble in cold water.

O-iso*Amylharmol*, thick rhombs, M.P. 236–237° (corr.). (Found: C, 76·4; H, 7·6 %. $C_{17}H_{20}ON_2$ requires C, 76·1; H, 7·5 %.) The *hydrochloride*, needles, M.P. 206–208° (corr.) after drying, soluble in 20 parts of water at 80°. Strong solutions gelatinise on cooling, but the gel soon passes into a mass of small crystals. Sparingly soluble in cold water. (Found, in air-dried salt: loss at 100°, nil; at 120°, 5·6 %. $C_{17}H_{20}ON_2$, HCl, H_2O requires H_2O, 5·6 %. Found, in dried salt: Cl, 11·9 %. $C_{17}H_{20}ON_2$, HCl requires Cl, 11·7 %.)

O-n-*Hexylharmol*, long needles, M.P. 143–144° (corr.). The *hydrochloride*, felted needles, M.P. 212–213° (corr.). (Found: Cl, 10·9 %. $C_{18}H_{22}ON_2$, HCl requires Cl, 11·1 %.)

O-n-*Heptylharmol*, large anhydrous leaflets, M.P. 131–132° (corr.) after sintering at 110°. (Found: C, 76·8; H, 8·1 %. $C_{19}H_{24}ON_2$ requires C, 77·0; H, 8·1 %.) The *hydrochloride*, needles, M.P. 228–229° (corr.). (Found: N, 8·3; Cl, 10·6 %. $C_{19}H_{24}ON_2$, HCl requires N, 8·4; Cl, 10·7 %.) The *methiodide*, minute glistening needles, M.P. 239–240° (corr.) gives on double decomposition with silver chloride in aqueous solution the *methochloride*, colourless tablets, M.P. 251–252° (corr.). This gives a bright but gelatinous 1 % solution in cold water. (Found: Cl, 10·1 %. $C_{20}H_{27}ON_2Cl$ requires Cl, 10·2 %.) It yields with ammonia O-n-*heptyl*-N-*methylharmol*, pale yellow needles, M.P. 227° (corr.) after darkening at 120°, insoluble in water, but very readily soluble in alcohol giving an intensely yellow solution. (Found: C, 77·5; H, 8·4 %. $C_{20}H_{26}ON_2$ requires C, 77·4; H, 8·4 %.)

O-n-*Octylharmol*, needles, M.P. 98–100° (corr.). (Found: C, 76·9; H, 8·4 %. $C_{20}H_{26}ON_2$ requires C, 77·3; H, 8·4 %.) The *hydrochloride*, thick needles, M.P. 217–218° (corr.) after drying. (Found, in air-dried salt: loss at 100°, 5·1 %. $C_{20}H_{26}ON_2$, HCl, H_2O requires H_2O, 4·9 %. Found, in dried salt: Cl, 10·1 %; $C_{20}H_{26}ON_2$, HCl requires Cl, 10·2 %.)

O-sec-*Octylharmol hydrochloride* (prepared by means of α-methylheptyl iodide), short prisms, M.P. 254–255° (corr.) after drying. (Found, in air-dried salt: loss at 100°, 11·6, 11·3; Cl, 10·4 %. $C_{20}H_{26}ON_2$, HCl, $2\frac{1}{2}H_2O$ requires H_{20}, 11·5; Cl, 10·2 %.) This salt is readily soluble in hot water, and the solution crystallises on cooling and does not set to a gel. The solubility in cold water is 1 in 28.

O-n-*Nonylharmol*, long needles, M.P. 115° (corr.). (Found: C, 77·5; H, 8·6 %. $C_{21}H_{28}ON_2$ requires C, 77·7; H, 8·6 %.) The *hydrochloride* crystallises from a mixture of alcohol and acetone in anhydrous leaflets, M.P. 205–207° (corr.). It is readily soluble in alcohol, and dissolves in 20 parts of water at 45° giving a turbid solution, which does not gelatinise on cooling but yields an amorphous deposit; after filtering, an opalescent solution is obtained containing about 1 part of the salt in 140. (Found: N, 7·7; Cl, 9·8 %. $C_{21}H_{28}ON_2$, HCl requires N, 7·8; Cl, 9·8 %.)

O-n-*Decylharmol*, needles, M.P. 105–107° (corr.). (Found: C, 78·1; H, 8·8 %. $C_{22}H_{30}ON_2$ requires C, 78·1; H, 8·9 %.) The *hydrochloride*, leaflets, M.P. 208° (corr.), after drying. (Found, in air-dried salt: loss at 100°, 2·4 %. $C_{22}H_{30}ON_2$, HCl, $\frac{1}{2}H_2O$ requires H_2O, 2·3 %. Found, in dried salt: Cl, 9·3 %. $C_{22}H_{30}ON_2$, HCl requires Cl, 9·5 %.)

O-iso*Decylharmol* (O-β-n-*Butyl*-n-*hexylharmol*) was prepared by means of β-n-*butyl*-n-*hexyl bromide* (B.P. 108–109°/14 mm.; *d*, 1·073 at 16°. Found: Br, 35·9 %. $C_{10}H_{21}Br$ requires Br, 36·2 %) which resulted from the action of hydrogen bromide upon β-n-*butyl*-n-*hexyl alcohol* (B.P. 213–216°), prepared by reducing ethyl di-*n*-butyl acetate with sodium and alcohol. *O-iso*Decylharmol, flocks of needles, M.P. 101–104° (corr.). (Found: C, 78·1; H, 8·9 %. $C_{22}H_{30}ON_2$ requires C, 78·1; H, 8·9 %.) The *hydrochloride*, needles containing $2H_2O$, M.P. hydrated 88–90°; anhydrous 210–212° (corr.). (Found: loss at 100°, 8·5 %. $C_{22}H_{31}ON_2Cl, 2H_2O$ requires H_2O, 8·8 %. Found, in dried salt: Cl, 9·6 %. $C_{22}H_{31}ON_2Cl$ requires Cl, 9·5 %.)

O-n-*Dodecylharmol*, needles, M.P. 119–120° (corr.). (Found: C, 78·5; H, 9·4 %. $C_{21}H_{34}ON_2$ requires C, 78·7; H, 9·3 %.) The *hydrochloride*, nacreous leaflets, M.P. 208·5° (corr.). (Found: Cl, 8·8 %. $C_{21}H_{34}ON_2$, HCl requires Cl, 8·8 %.)

O-*Benzylharmol*, minute needles, M.P. 213° (corr.) after sintering from 195°. (Found: C, 79·2; H, 5·6 %. $C_{19}H_{16}ON_2$ requires C, 79·2; H, 5·6 %.) The *hydrochloride*, needles, M.P. 256–257° (corr.) gives a 2 % solution in hot water, which gelatinises on cooling. (Found: Cl, 11·2 %. $C_{19}H_{16}ON_2$, HCl requires Cl, 11·0 %.)

O-ω-*Hydroxy*-n-*decylharmol*, prepared from ακ-decylenechlorohydrin, formed needles, M.P. 174–175° (corr.). (Found: C, 74·5; H, 8·5 %. $C_{22}H_{30}O_2N_2$ requires C, 74·6; H, 8·5 %.) The *hydrochloride*, needles, M.P. 180° (corr.) after drying is sparingly soluble in water. (Found, in air-dried salt: loss at 100°, 4·2 %. $C_{22}H_{30}O_2N_2$, HCl, H_2O requires H_2O, 4·4 %. Found, in dried salt, Cl, 9·1 %. $C_{22}H_{30}O_2N_2$, HCl requires Cl, 9·1 %.) The lactate is soluble in 10 parts of hot water, but separates in gelatinous masses on cooling.

ω-*Dialkylaminoalkyl ethers of harmol.*

These were prepared by boiling alcoholic solutions of harmol (1 mol.), aqueous (5*N*) sodium hydroxide (2 mols.), and the appropriate ω-dialkylaminoalkyl chloride hydrochloride (1 mol.). Substances of the latter class were made by the action of thionyl chloride in chloroform upon the corresponding ω-dialkylaminoalkyl alcohols [Gough and King, 1928]. Except in the case of the undecyl derivatives these alcohols were prepared by heating dialkylamines with the corresponding chlorohydrins. Several of the latter were prepared in a crude state by boiling the appropriate glycol with concentrated hydrochloric acid, but were contaminated with much of the corresponding dichlorides, so that on treatment with dialkylamines mixtures of ω-dialkylaminoalkyl alcohols and bis-dialkylaminoparaffins resulted. On the appearance of Bennett and Mosses's work [1931] the chlorohydrins were made in a purer state by their method. These reactions may be outlined as follows:

$$\begin{tikzcd}
\mathrm{HO(CH_2)}_n\mathrm{OH} \arrow[r] & \mathrm{HO(CH_2)}_n\mathrm{Cl} \arrow[dl] & {[\rightarrow \mathrm{Cl(CH_2)}_n\mathrm{Cl}]} \arrow[d] \\
\mathrm{HO(CH_2)}_n.\mathrm{NRR'} \arrow[r] & \mathrm{Cl(CH_2)}_n.\mathrm{NRR'} & {[\mathrm{RR'N.(CH_2)}_n.\mathrm{NRR'}]}
\end{tikzcd}$$

The λ-dialkylaminoundecyl chlorides were made by a different method. Ethyl κ-bromoundecoate was condensed with dialkylamines, and the amino-esters reduced to the amino-alcohols which were then treated with thionyl chloride.

$$\mathrm{Br(CH_2)_{10}.CO_2Et} \rightarrow \mathrm{RR'N.(CH_2)_{10}.CO_2Et} \rightarrow \mathrm{RR'N(CH_2)_{11}.OH}.$$

ω-*Dialkylaminoalkyl alcohols.*

η-*Diethylaminoheptyl alcohol.* η-Chloroheptyl alcohol (39 g.) and diethylamine (39 g.) were heated under reflux at 70° for 48 hours, more diethylamine being added from time to time to replace that lost by evaporation. The solid mass was mixed with aqueous sodium hydroxide and extracted with ether. This was distilled into hydrochloric acid to recover diethylamine, and the non-volatile residue was separated by means of acid into unchanged chlorohydrin (8·4 g.) and η-diethylaminoheptyl alcohol (32·7 g. = 90 % on chlorohydrin used up; B.P. 139–141°/25 mm.). (Found: C, 70·7; H, 13·4 %. $C_{11}H_{25}ON$ requires C, 70·6; H, 13·3 %.)

The following alcohols were prepared similarly:

θ-*Diethylaminooctyl alcohol*, yield 89 %, B.P. 187–190°/72 mm. (Found: C, 71·5; H, 13·5 %. $C_{12}H_{27}ON$ requires C, 71·7; H, 13·4 %.)

ι-*Diethylaminononyl alcohol*, yield 72 %, B.P. 171–173°/23 mm. (Found: C, 72·6; H, 13·6 %. $C_{13}H_{29}ON$ requires C, 72·6; H, 13·5 %.)

κ-*Dimethylaminodecyl alcohol*, yield 64 %, B.P. 202–204°/30 mm. (Made in a sealed tube at 100°.) (Found: C, 71·6; H, 13·4 %. $C_{12}H_{27}ON$ requires C, 71·7; H, 13·4 %.)

κ-*Diethylaminodecyl alcohol*, yield 80 %, B.P. 193–196°/50 mm. (Found: C, 73·5; H, 13·4 %. $C_{14}H_{31}ON$ requires C, 73·3; H, 13·5 %.) The *picrate* had M.P. 145°.

κ-*Dibutylaminodecyl alcohol*, yield 91 %, B.P. 233–235°/50 mm. (Found: C, 75·6; H, 13·7 %. $C_{18}H_{39}ON$ requires C, 75·7; H, 13·7 %.)

*κ-Di-*n*-amylaminodecyl alcohol,* yield 62 %, B.P. 227–229°/17 mm. (Found: C, 76·6; H, 13·8 %. $C_{20}H_{43}ON$ requires C, 76·7; H, 13·8 %.)

κ-Piperidyldecyl alcohol, yield 89 %, B.P. 210–211°/25 mm., M.P. 55–56°. (Found: C, 74·7; H, 12·7 %. $C_{15}H_{31}ON$ requires C, 74·7; H, 12·7 %.)

λ-Diethylaminoundecyl alcohol. Ethyl κ-bromoundecoate (147 g.) and diethylamine (80 g.) were heated under reflux for 2 days. The product was worked up similarly to the products of the interaction of diethylamine with the chlorohydrins, and gave *ethyl κ-diethylaminoundecoate* (103 g. = 73 %, B.P. 210–212°/50 mm.). (Found: C, 71·4; H, 12·2 %. $C_{17}H_{35}O_2N$ requires C, 71·6; H, 12·3 %.) On reducing the amino-ester with sodium and alcohol under the conditions employed by Bennett and Mosses [1931] for reducing esters of dicarboxylic acids to glycols, *λ-diethylaminoundecyl alcohol* was obtained in 71 % yield, B.P. 219–220°/72 mm. (Found: C, 73·9; H, 13·8 %. $C_{15}H_{33}ON$ requires C, 74·1; H, 13·6 %.) *Ethyl κ-di-*n*-butylaminoundecoate,* yield 75 %, B.P. 210°/25 mm. (Found: C, 73·8; H, 12·6 %. $C_{21}H_{43}O_2N$ requires C, 73·8; H, 12·6 %), similarly gave on reduction *λ-di-*n*-butylaminoundecyl alcohol,* yield 62 %, B.P. 215°/17 mm. (Found: C, 76·2; H, 13·8 %. $C_{19}H_{41}ON$ requires C, 76·2; H, 13·7 %) and *ethyl κ-di-*n*-heptylaminoundecoate,* yield 78 %, B.P. 246–250°/4 mm. (Found: C, 76·1; H, 12·9 %. $C_{27}H_{55}O_2N$ requires C, 76·3; H, 13·1 %) gave *λ-di-*n*-heptylaminoundecyl alcohol,* yield 70 %, B.P. 236–238°/4 mm. (Found: C, 78·3; H, 13·9 %. $C_{25}H_{53}ON$ requires C, 78·5; H, 13·8 %.)

ω-Dialkylaminoalkyl chlorides.

These were prepared by the method of Gough and King [1928] and isolated as hydrochlorides in yields of 80–100 %. The lower members of the series separated from absolute alcohol or mixtures of absolute alcohol with anhydrous ether in colourless needles, usually anhydrous, which were very readily soluble in water, alcohol or acetone, but insoluble in dry ether.

η-Diethylaminoheptyl chloride hydrochloride, contains $\frac{1}{2}H_2O$, and has M.P. (hydrated) 82–84°; dried *in vacuo* 76–78°. (Found, in air-dried salt: loss *in vacuo,* 3·3 %. $C_{11}H_{24}NCl, \frac{1}{2}H_2O$ requires H_2O, 3·6 %. Found, in salt dried *in vacuo*: C, 52·3; H, 10·5; Cl, 29·0 %. $C_{11}H_{24}NCl$, HCl requires C, 52·6; H, 10·4; Cl, 29·3 %.)

θ-Diethylaminooctyl chloride hydrochloride, M.P. 73°. (Found: N, 5·4; Cl, 27·5 %. $C_{12}H_{26}NCl$, HCl requires N, 5·5; Cl, 27·7 %.)

ι-Diethylaminononyl chloride hydrochloride, M.P. 85–86° (corr.).

κ-Dimethylaminodecyl chloride hydrochloride, M.P. 136–138° (corr.). (Found: Cl, 27·6 %. $C_{12}H_{26}NCl$, HCl requires Cl, 27·7 %.)

κ-Diethylaminodecyl chloride hydrochloride, M.P. 80–82° (corr.). (Found: C, 59·1; H, 11·1; Cl, 24·8 %. $C_{14}H_{20}NCl$, HCl requires C, 59·2; H, 10·9; Cl, 25·0 %.)

κ-Piperidyldecyl chloride hydrochloride, M.P. 127–128° (anhyd.). (Found: C, 60·8; H, 10·6; Cl, 23·9 %. $C_{15}H_{30}NCl$, HCl requires C, 60·8; H, 10·45; Cl, 24·0 %.)

λ-Diethylaminoundecyl chloride hydrochloride, M.P. 84–86° (corr.). (Found: C, 60·2; H, 11·05 %. $C_{15}H_{32}NCl$, HCl requires C, 60·3; H, 11·05 %.)

κ-Di-*n*-butylaminodecyl chloride hydrochloride, κ-di-*n*-amylaminodecyl chloride hydrochloride, λ-di-*n*-butylaminoundecyl chloride hydrochloride and λ-di-*n*-heptylaminoundecyl chloride hydrochloride were obtained—and used as alkylating agents—in the form of syrups, which did not crystallise.

Tetraethyldiaminoalkylenes.

In two preliminary experiments chlorohydrins heavily contaminated with the corresponding dichlorides were caused to react with diethylamine and the resulting crude amino-alcohols were treated with thionyl chloride. The crude mixtures of diethylaminoalkyl chloride hydrochloride and tetraethyldiaminoalkylene dihydrochloride were dissolved in absolute alcohol and could be separated sharply by fractional precipitation with ether, the diamino-derivative precipitating last.

η-Tetraethyldiaminoheptane dihydrochloride, colourless hygroscopic crystals (containing $1H_2O$). (Found: H_2O, 5·6 %. Calc.: H_2O, 5·4 %), M.P. 226° (corr.), after drying at 105° was readily soluble in water or alcohol, but insoluble in ether. (Found, in dried salt: C, 56·9; H. 11·5; Cl, 22·9 %. $C_{15}H_{34}N_2$, 2HCl requires C, 57·2; H, 11·4; Cl, 22·6 %.)

κ-Tetraethyldiaminodecane dihydrochloride, colourless hygroscopic needles, M.P. 210–212° (corr.). (Found: Cl, 19·7 %. $C_{18}H_{40}N_2$, 2HCl requires Cl, 20·0 %.)

O-*ω-Dialkylaminoalkylharmols.*

These were prepared as indicated upon p. 735. They were isolated as dihydrochlorides, which crystallised in colourless needles, more or less readily soluble in water yielding neutral solutions which did not gelatinise. The corresponding bases were obtained by adding ammonia to hot dilute alcoholic solutions of the dihydrochloride, and crystallised from alcohol in colourless needles. Most of those which were analysed contained $2H_2O$.

O-*β-Diethylaminoethylharmol*, M.P. 167–168° (corr.); the *dihydrochloride*, M.P. 295° (corr. decomp.). (Found: C, 51·0; H, 7·4; N, 9·9; Cl, 16·8 %. $C_{18}H_{23}ON_3$, 2HCl, $3H_2O$ requires C, 50·9; H, 7·3; N, 9·9; Cl, 16·7 %.)

O-*η-Diethylaminoheptylharmol*, M.P. 109–111° (corr.); the *dihydrochloride* crystallises from a mixture of alcohol and acetone in thick needles containing $3H_2O$, of which 2 mols. are lost in a vacuum over H_2SO_4 and the third at 100°. The air-dried salt has M.P. 144–146° (corr.); the dried salt has M.P. 138–140°. (Found, in air-dried salt: loss in a vacuum, 6·4; total loss at 100°, 10·7; C, 56·2; H, 8·32 %. $C_{23}H_{33}ON_3$, 2HCl, $3H_2O$ requires $3H_2O$, 10·9; C, 56·0; H, 8·32 %. Found, in dried salt: Cl, 16·2 %. $C_{23}H_{33}ON_3$, 2HCl requires Cl, 16·2 %.)

O-*θ-Diethylaminooctylharmol*, M.P. 98–99° (corr.); contains $2H_2O$. (Found: C, 69·3; H, 9·36 %. $C_{24}H_{35}ON_3$, $2H_2O$ requires C, 69·0; H, 9·34 %.) The *dihydrochloride* crystallises from aqueous acetone with $1\frac{1}{2}H_2O$ which is lost at 80°. The air-dried salt has M.P. 97–100°, and the dried salt 88°. It is soluble in 8 parts of cold water. (Found, in air-dried salt: loss at 80°, 5·4 %. $C_{24}H_{35}ON_3$, 2HCl, $1\frac{1}{2}H_2O$ requires H_2O, 5·6 %. Found, in dried salt: Cl, 15·5 %. $C_{24}H_{35}ON_3$, 2HCl requires Cl, 15·6 %.)

O-*ι-Diethylaminononylharmol*, M.P. 92° (corr.); its *dihydrochloride* crystallises from dilute hydrochloric acid with $3H_2O$, which is lost at 100°. M.P. (air-dried) 162–167°; (anhydrous) 168° (corr.). It is soluble in 30 parts of cold water. (Found, in air-dried salt: loss at 100°, 10·3; C, 57·6; H, 8·66 %. $C_{25}H_{37}ON_3$, 2HCl, $3H_2O$ requires H_2O, 10·5; C, 57·5; H, 8·63 %. Found, in dried salt: Cl, 15·1 %. $C_{25}H_{37}ON_3$, 2HCl requires Cl, 15·3 %.)

O-*κ-Dimethylaminodecylharmol*, M.P. 91–94° (corr.); its *dihydrochloride*, needles from alcohol, M.P. 180–182° (corr.) contain $2\frac{1}{2}H_2O$ which is lost at 100°, soluble in 18 parts of cold water. (Found, in air-dried salt: loss at 100°, 9·08; C, 57·5; H, 8·62 %. $C_{24}H_{35}ON_3$, 2HCl, $2\frac{1}{2}H_2O$ requires H_2O, 9·02; C, 57·8; H, 8·43 %. Found, in dried salt: Cl, 15·6 %. $C_{24}H_{35}ON_3$, 2HCl requires Cl, 15·7 %.)

O-*κ-Diethylaminodecylharmol*, M.P. (air-dried); 117–118° (corr.); (anhydrous) 137° (corr.). (Found, in air-dried substance: loss *in vacuo* over P_2O_5, 7·9 %. $C_{26}H_{39}ON_3$, $2H_2O$ requires H_2O, 8·1 %.) Its *dihydrochloride* crystallises from alcohol-acetone with $1H_2O$, which is lost at 100°. M.P. (air-dried) 134–135°; (anhydrous) 149–150° (corr.). It is soluble in about 5 parts of cold water. (Found, in air-dried salt: loss at 100°, 3·4 %. $C_{26}H_{39}ON_3$, 2HCl, H_2O requires H_2O, 3·6 %. Found, in dried salt: C, 64·8; H, 8·6; N, 8·5; Cl, 14·7 %. $C_{26}H_{39}ON_3$ HCl requires C, 64·8; H, 8·5; N, 8·7; Cl, 14·2 %.)

O-*κ-Di*-n-*butylaminodecylharmol*, M.P. 59–60° (corr.), contains $2H_2O$. (Found: C, 72·0; H, 10·0 %. $C_{30}H_{47}ON_3$, $2H_2O$ requires C, 71·9; H, 10·2 %.) The *dihydrochloride* crystallises from alcohol-acetone in needles, which rapidly darken in moist air. The vacuum-dried salt melts at 98–99° and contains $1\frac{1}{2}H_2O$ which is lost at 80°. It is soluble in 13 parts of cold water. (Found: loss, 4·9 %. $C_{30}H_{47}ON_3$, 2HCl, $1\frac{1}{2}H_2O$ requires H_2O, 4·8 %. Found, in dried salt: Cl, 13·0 %. $C_{30}H_{47}ON_3$, 2HCl requires Cl, 13·2 %.)

O-*κ-Piperidyldecylharmol*, M.P. 109–111° (corr.). (Found: C, 76·6; H, 9·3 %. $C_{27}H_{39}ON_3$ requires C, 76·7; N, 9·2 %.) The *dihydrochloride* crystallises from water with $4H_2O$ which is lost at 100°. M.P. (air-dried) 116–118°; (anhydrous) 117–118°. It is soluble in 200 parts of cold water. (Found, in air-dried salt: loss at 100°, 12·45; Cl, 12·45 %. $C_{27}H_{39}ON_3$, 2HCl, $4H_2O$ requires H_2O, 12·7; Cl, 12·55 %.)

O-*λ-Diethylaminoundecylharmol*, M.P. 82–84° (corr.), contains $2H_2O$. (Found: C, 70·4; H, 9·9 %. $C_{27}H_{41}ON_3$,$2H_2O$ requires C, 70·5; H, 9·8 %.) The *dihydrochloride* crystallises from alcohol-acetone with $1H_2O$, which is lost at 80°, M.P. (air-dried) 94–97°; (anhydrous) 99–101° (corr.).

It is soluble in 3 parts of cold water. (Found, in air-dried salt: loss at 80°, 3·7; Cl, 13·8 %. $C_{27}H_{41}ON_3$,2HCl, H_2O requires H_2O, 3·6; Cl, 13·8 %.)

λ-*Di*-n-*butylaminoundecylharmol*, M.P. 65–67° (corr.); its *dihydrochloride* crystallises from water with $3H_2O$, of which two are lost at 100° and the rest at 115°. M.P. (air-dried) 138° (corr.), sinters at 135°; (anhydrous) 138–139° (corr.). It is soluble in 80 parts of cold water. (Found, in air-dried salt: loss at 115°, 9·17 %. $C_{31}H_{54}ON_3$, 2HCl,$3H_2O$ requires 6·68 and 9·22 % for loss of $2H_2O$ and $3H_2O$ respectively. Found, in dried salt, Cl, 12·9 %. $C_{31}H_{54}ON_3$,2HCl requires Cl, 12·85 %.)

Alkylharmalols.

These were prepared by the action of alkyl bromides on harmalol in aqueous-alcoholic sodium hydroxide (1 mol.) and worked up in the same way as the alkylharmols.

O-*Ethylharmalol* was precipitated by ammonia from an alcoholic solution of the hydrochloride in pale brown needles, M.P. 237–239° (corr.). (Found: C, 73·7; H, 7·1 %. $C_{14}H_{16}ON_2$ requires C, 73·7; H, 7·0 %.) The *hydrochloride* crystallises from 5 parts of hot water in bright yellow needles containing $1H_2O$, which is lost at 100°. M.P. (hydrated) 265–268° (corr.); (anhydrous) 263–264° (corr.). It is soluble in 30 parts of cold water. (Found, in air-dried salt; loss at 100°, 6·1 %. $C_{14}H_{16}ON_2$, HCl, H_2O requires H_2O, 6·4 %. Found, in dried salt: Cl, 13·4 %. $C_{14}H_{16}ON_2$, HCl requires Cl, 13·4 %.)

O-n-*Propylharmalol* crystallises from moist alcohol in long brown flattened needles, M.P. 195–197° (corr.). (Found: C, 74·5; H, 7·6 %. $C_{15}H_{18}ON_2$ requires C, 74·4; H, 7·4 %.) The *hydrochloride* crystallises from water or moist alcohol in pale yellow needles, M.P. 232–234° (corr.). It is soluble in 9 parts of cold water. (Found: Cl, 12·7 %. $C_{15}H_{18}ON_2$, HCl requires Cl, 12·8 %.)

O-n-*Butylharmalol* crystallises from alcohol in colourless slender needles, M.P. 173° (corr.). (Found: C, 75·0; H, 7·9 %. $C_{16}H_{20}ON_2$ requires C, 75·0; H, 7·8 %.) The *hydrochloride* crystallises from water or alcohol in minute yellow needles, M.P. 213–214° (corr.). It is soluble in 70 parts of water at 35°. (Found: Cl, 12·3 %. $C_{15}H_{20}ON_2$, HCl requires Cl, 12·2 %.)

SUMMARY.

1. A convenient method for preparing harmol and harmalol is described.

2. A long series of *O*-alkyl and substituted *O*-alkyl ethers of harmol has been prepared by the alkylation of harmol.

3. In the *n*-alkyl series, the peak of bactericidal activity is found at *n*-butyl- for *B. typhosus* and at *n*-amyl- for *S. aureus*, whilst the peak of amoebicidal activity is found at *n*-nonyl-harmol.

4. In the ω-diethylaminoalkyl series, the peak of bactericidal activity to *B. typhosus* is found at ω-diethylaminononylharmol.

5. Several members of these series were tested for antimalarial or trypanocidal activity, but none gave a positive result.

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XCVII. PHENYL *ISO*CYANATE PROTEIN COMPOUNDS AND THEIR IMMUNOLOGICAL PROPERTIES.

By SYDNEY JOHN HOPKINS and ARTHUR WORMALL.

From the Department of Physiology, the University of Leeds.

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THE chemistry of antigens can be studied in several different ways, and each method has its own definite advantages. On one hand, the protein molecule can be altered by the action of enzymes, acids or alkalis, or chemical alteration may be effected by nitration, iodination, oxidation, *etc.*, and a study of protein specificity made. Another method [Landsteiner and Lampl, 1917; 1918; Landsteiner, 1920; Landsteiner and van der Scheer, 1927; 1929] has involved the attachment to proteins of groupings of well-defined chemical nature by the interaction of various diazo-compounds with the protein to give azo-proteins, and this second method has been utilised for a more general study of the relationship between antigenic specificity and chemical structure. Full discussions of the results of the application of these and other methods can be found elsewhere [Hartley, 1931; Landsteiner, 1927; 1933; Wells, 1929].

The experiments with the azo-proteins have yielded results of great value and have very materially increased our knowledge about the specificity of antigens. It would appear desirable, therefore, that other methods for introducing into the protein molecule new groupings of definite structure should be utilised, but suitable methods of this nature are not readily found. Any treatment which is at all drastic and which involves the use of strong acids, alkalis or oxidising agents may be objected to on the grounds that hydrolytic and other changes necessarily accompany the introduction of new groups. The ideal method would be one which resulted in the introduction of a determinable number of new groups into a definite part of the protein molecule unaccompanied by any other change (hydrolytic, oxidative, *etc.*). With this object in view we have investigated the reaction between proteins and phenyl *iso*cyanates. Raper [1907] prepared phenyl *iso*cyanate derivatives of various fractions of the peptones from serum-albumin and obtained well-defined products with characteristic melting-points. The conclusion was reached by Raper that these compounds are definite chemical individuals. More recently, Folley [1932] has prepared the phenyl *iso*cyanate derivative of plastein. There appeared to be no definite evidence, however, that native proteins could react with phenyl *iso*cyanate in this way, although it seemed most probable that a reaction of this type would take place. Preliminary experiments were therefore carried out to determine whether phenyl *iso*cyanate would react with proteins such as serum-globulin and caseinogen. Since the main object of this investigation was concerned with the immunological study of these protein compounds, certain slight modifications had to be introduced into the method mainly with the idea of maintaining during the reaction a p_H between 7 and 8. The experiments were successful in yielding new protein derivatives which were strongly antigenic and which gave interesting results from the immunological view-point.

In the description below, this investigation is divided into two parts, the first part dealing with the preparation of these phenylureido-compounds and the study of their chemical properties, and the second part with the immunological experiments.

I. THE PREPARATION AND PROPERTIES OF THE PHENYLUREIDO-PROTEINS.

Preparation of phenylureido-caseinogen.

"Light white caseinogen" (B.D.H.) was purified by precipitation from an alkaline solution by the addition of acetic acid. 30 g. of caseinogen were made into a smooth paste with about 500 cc. of distilled water, dilute NaOH added to bring the reaction between p_H 9 and 10 and the solution centrifuged. The caseinogen was precipitated from the supernatant solution by means of dilute acetic acid and filtered off. The precipitate was dissolved in about 500 cc. of distilled water with the aid of dilute NaOH, the p_H being kept below 10, and, after the solution had been centrifuged to remove the undissolved matter, the caseinogen was precipitated again with acetic acid. The final product was taken up in about 500 cc. of distilled water, dilute NaOH added to give p_H 9 and the solution centrifuged.

To 50 cc. of this purified caseinogen solution were added 100 cc. of a phosphate buffer of p_H 8·0 (250 cc. of 0·2 *M* KH_2PO_4 *plus* 234 cc. of 0·2 *N* NaOH, made up to 1 litre). The mixture was then cooled in ice and 2 cc. of phenyl *iso*cyanate added slowly with continuous stirring. The reaction of the fluid was determined at frequent intervals by means of indicators, and 0·2 *N* NaOH was added when necessary to restore the p_H to 8·0 approximately. After having been stirred for about 1 hour, the mixture was adjusted to p_H 8·0 again and centrifuged, the precipitate being discarded. 2 % acetic acid was then added to the solution to give maximum formation of a flocculent precipitate. This product was removed by centrifuging and redissolved in about 200 cc. of distilled water with the addition of sufficient 0·2 *N* NaOH to give a clear solution. When this and similar precipitates were dissolved in water with the aid of dilute NaOH, the alkali was added very cautiously and frequent tests were made to ensure that the reaction of the solution did not become more alkaline than about p_H 8·5. This precaution was taken in order that there might not be any significant loss of antigenic power due to the action of alkali. At p_H 11, the relatively labile serum-proteins appear to suffer little loss of antigenic power at about room temperatures over a period of several hours [Johnson and Wormall, 1932], and since the solutions referred to in this paper were rarely allowed, even for a short period, to become more alkaline than p_H 8·5 and never more alkaline than p_H 9·5, it might justifiably be concluded that no loss of antigenic property or change in specificity occurred as a result of the action of alkali.

The phenylureido-caseinogen was reprecipitated by the addition of dilute acetic acid, the precipitate collected by centrifuging and redissolved as before. A third precipitation was made, and the precipitate finally dissolved in 30 to 40 cc. of distilled water with the addition of just sufficient 0·2 *N* NaOH to give a clear solution at about p_H 7·6.

Preparation of phenylureido-gelatin.

1 g. of gelatin (Coignet's "Gold Label") was dissolved in a warmed mixture of 65 cc. of distilled water and 65 cc. of the phosphate buffer of p_H 8·0. The solution was then cooled in ice, 0·5 cc. of phenyl *iso*cyanate was added slowly

and the whole stirred for about one hour, the reaction of the mixture being maintained at about p_H 8·0 by the addition of 0·2 *N* NaOH as above. At the end of this period the insoluble matter was discarded and the phenylureido-gelatin in the supernatant solution obtained as a gelatinous precipitate by the addition of acetic acid. Purification of this product was effected as for the corresponding caseinogen compound by two further precipitations with acetic acid. In the preparation of this gelatin compound and in that of *p*-bromophenylureido-gelatin, the precipitates dissolved in water at p_H 7·5 to 8·0 with some difficulty in the cold, but solution was readily effected at 30 to 35°. The solutions were not allowed to remain at these higher temperatures longer than was absolutely necessary.

Preparation of phenylureido-horse serum-globulin.

The serum-globulins were separated as follows. 100 cc. of horse serum were diluted with about 900 cc. of distilled water, the solution cooled in ice and CO_2 passed in to give maximum precipitation. This suspension was then allowed to stand in the ice-chest for 24 to 48 hours, the supernatant fluid syphoned off, and the globulin precipitate separated by centrifuging the remainder of the fluid. This precipitate was then dissolved in about 100 cc. of 0·9 % NaCl solution, 0·2 *N* NaOH being added to adjust the p_H to about 7·5, excess of alkali being carefully avoided. From this solution the globulin was again precipitated by CO_2, the suspension allowed to stand for 24 to 48 hours in the ice-chest and the centrifuged deposit dissolved in about 40 cc. of 0·9 % NaCl solution, the reaction of the final solution being adjusted to about p_H 7·5.

To 100 cc. of this solution of horse serum-globulin were added 100 cc. of phosphate buffer of p_H 8 and 10 cc. of 10 % NaCl solution (added to maintain the concentration of NaCl at approximately 0·9 %). The mixture was cooled in ice and 3·5 cc. of phenyl *iso*cyanate added slowly to the well-stirred solution. The reaction of the mixture was maintained as before at about p_H 8·0, and after one hour 200 cc. of 0·9 % NaCl were added, and the fluid was centrifuged. The precipitate was washed twice with about 100 cc. of 0·9 % NaCl solution with 0·2 *N* NaOH added to give p_H 8, and the washings were added to the original supernatant solution. (This washing of the precipitate was only carried out when it was found desirable to improve the yield of phenylureido-protein.) The phenylureido-globulin was then precipitated by acetic acid and purified by two further precipitations with acetic acid as described for phenylureido-caseinogen, the precipitate being dissolved in 0·9 % NaCl solution each time instead of in distilled water: the solution of the globulin preparation was never allowed to become more alkaline than p_H 8·5. The final product was dissolved in about 70 cc. of 0·9 % NaCl solution with sufficient 0·2 *N* NaOH to give p_H 7·5.

Preparation of p-*bromophenylureido-protein compounds.*

Similar *p*-bromophenylureido-compounds of caseinogen, gelatin and horse serum-globulin were made by the action of *p*-bromophenyl *iso*cyanate in ethereal solution on a solution of the protein in a phosphate buffer at p_H 8.

100 cc. of caseinogen solution, containing approximately 3·5 g. of twice reprecipitated caseinogen (see above) were mixed with 200 cc. of phosphate buffer of p_H 8·0, and the mixture was cooled in ice. A filtered solution of 2·5 g. of *p*-bromophenyl *iso*cyanate in about 30 cc. of ether was then added and the cooled mixture stirred vigorously for about 1½ hours. The p_H was maintained at about 8·0, and *p*-bromophenylureido-caseinogen was precipitated and purified

by two further precipitations as described for the phenylureido-compounds. Similar *p*-bromophenylureido-compounds of gelatin and horse serum-globulin were made.

In order that determinations of the bromine content might be made, it was necessary in some cases to have dried and purified preparations of these *p*-bromophenylureido-compounds. For this purpose a portion of the solution of the thrice precipitated preparation was treated with three times its volume of absolute alcohol and sufficient dilute acetic acid added to give maximum precipitation. The precipitate was then washed with absolute alcohol, an alcohol-ether mixture and finally with ether. This preparation was then dried *in vacuo* over $CaCl_2$, powdered and extracted with ether in a Soxhlet apparatus for 100 hours. The material was powdered again and extracted with ether for a further 100 hours. The final product was dried *in vacuo*, and bromine estimations were made by the Carius method, the following values being obtained:

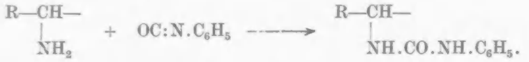
p-bromophenylureido-caseinogen:	4·1 % Br (1st preparation)
	5·0 % Br (2nd preparation)
p-bromophenylureido-gelatin:	2·6 % Br (1st preparation)
	2·2 % Br (2nd preparation)

Nitrogen determinations. Total nitrogen determinations were made by the micro-Kjeldahl method. The amount of *amino-nitrogen* present in the various solutions was determined (*a*) by the method of Van Slyke (micro-apparatus), and (*b*) by Sørensen's formaldehyde titration. For the last-named determination, the solution was adjusted to p_H 7 (neutral red range), neutralised formaldehyde solution added, and the amount of 0·02 or 0·1 *N* NaOH required to bring the solution to p_H 9 (phenolphthalein range) was then measured.

DISCUSSION.

By the method described above, a reaction has been induced between phenyl *iso*cyanate and native proteins, similar to that between certain peptone fractions and phenyl *iso*cyanate described by Raper [1907]. For the purposes of the investigation described here it was necessary to prevent gross changes in the p_H of the solution, *i.e.* to avoid, if possible, the use of strong alkali and to ensure that the acid produced during the reaction did not cause any marked acidity. After preliminary investigations it was found that a phosphate buffer of p_H 8·0 was admirably suitable for this purpose, especially if 0·2 *N* NaOH was added at intervals during the reaction in order to maintain the p_H at about 8·0. With these modifications the reaction appears to be ideal for immunological purposes, since a chemical change in the protein molecule is effected rapidly in approximately neutral solutions and at relatively low temperatures (4 to 8°). After being cooled and stirred for about one hour, the mixture of phenyl *iso*cyanate, protein and phosphate buffer contained some solid diphenylurea which could be largely removed by centrifuging, leaving a solution which contained a protein precipitable by dilute acetic acid at about p_H 4 to 4·5. This conversion of proteins such as the serum-proteins and gelatin which are not normally precipitated to any significant extent on the addition of dilute acetic acid (provided sodium chloride or some similar salt is present to prevent precipitation of the serum-globulin) into compounds which are readily precipitated by dilute acids suggests a chemical alteration in the protein molecule. Changes such as denaturation might possibly account, however, for this difference in solubility and it was considered desirable that further information about the action of phenyl *iso*cyanate on proteins should be obtained. Phenyl *iso*cyanate readily reacts with amino-acids in alkaline solution and will also react with peptones, as shown by Raper [1907].

The reaction between phenyl *iso*cyanate and proteins would appear to be concerned mainly with the free amino-groups of the protein molecule to give a reaction of the following type:



$$\underset{\substack{|\\ NH_2}}{R{-}CH{-}} + OC{:}N.C_6H_5 \longrightarrow \underset{\substack{|\\ NH.CO.NH.C_6H_5.}}{R{-}CH{-}}$$

Such a reaction would result in a diminution in the amount of free amino-nitrogen, and, assuming that no other change occurs, this diminution would be a measure of the extent of the reaction and the number of new groupings introduced. The results of some of the determinations of free amino-N, calculated as the percentage of total N present as free amino-N, are given in Table I, and from these figures it will be seen that there is a very considerable loss of free amino-N when these proteins are acted upon by phenyl and *p*-bromophenyl *iso*cyanates. Indeed, with caseinogen about 80 to 90 % of the free amino-N disappears when the protein has reacted with these *iso*cyanates.

The figures given in Table I do not show in every instance a very marked agreement between the results obtained with the two different methods for amino-N determination, but this disagreement is in accord with the observations of Wilson [1923] who compared these two methods using solutions of native and derived proteins. Wilson reached the conclusion that the method of Sørensen seems to be less susceptible to error than is Van Slyke's method in the analysis of proteins. From Table I it will be seen that the values given by Van Slyke's method were usually lower than those by the formaldehyde method, and these lower results with the former method are probably mainly due to precipitation of protein by the acid used in the determination.

Table I. *Amount of free amino-N in some proteins and their phenylureido- and* p-*bromophenylureido-derivatives.*

Recorded as the % of total N present as free amino-N.

Protein	Preparation	Method of amino-N determination	Unchanged protein %	Phenyl-ureido-protein %	*p*-Bromo-phenyl-ureido-protein %
Horse serum-globulin	1	Van Slyke	3·8	1·3	—
	2	Van Slyke	4·9	2·4	0·2
Caseinogen	1	Van Slyke	5·3	0·4	0·6
		Sørensen	5·5	0·6	0·7
	2	Van Slyke	4·4	0·6	0·1
		Sørensen	6·1	1·3	0·6
Gelatin	1	Van Slyke	2·3	1·7	0·5
		Sørensen	2·85	0·8	0·6
	2	Sørensen	3·0	0·8	1·2

This invariable diminution in free amino-N would appear to show that a reaction occurs between the phenyl *iso*cyanate and the free amino-groups of the protein molecule, but this evidence would not by itself be completely satisfactory, nor do the results exclude the possibility of additional types of reaction. The proof that one type of reaction only occurs, involving the formation of phenylureido-groupings, will be more complete if it can be shown that the number of new groups entering the protein molecule is equal to the number of free amino-groups which have disappeared. Attempts to correlate these two have been made with the *p*-bromophenyl *iso*cyanate protein compounds, with fairly satisfactory results. From the diminution in free amino-N (Sørensen's method) when caseinogen is converted into the *p*-bromophenylureido-compound, and using the value 15·62 % for the N content of caseinogen [Plimmer, 1917, p. 131], it was calculated that one sample of the dried *p*-bromophenylureido-caseinogen obtained should have 3·9 % of bromine; the observed value was 4·1 %. A second sample of this compound gave "calculated" and observed values of 4·4 and 5·0 % respectively. With the gelatin preparations, samples of dried *p*-bromophenylureido-gelatin which should have contained 1·8 and 2·4 % of bromine (from the diminution in amino-N by the formaldehyde method and taking the figure 18·00 % for the N content of gelatin [Plimmer, p. 131]) gave values of 2·2 and 2·6 % respectively. This agreement may be considered quite satisfactory, and thus these results appear to show that the only reaction concerned is that between the *p*-bromophenyl *iso*cyanate and the free amino-groups. The observed values are somewhat higher than the values calculated from the amino-N diminution, but the divergence is not sufficient to indicate the introduction of bromine-containing groups into other parts of the protein molecule. The small differences might easily be due to the presence of other bromine-containing substances (di-*p*-bromophenylurea, *etc.*) in the dried *p*-bromophenylureido-proteins, in spite of the fact that continuous extraction with ether, with occasional repowdering of the material, was carried out for over 200 hours.

The chemical evidence appears, therefore, to show that a reaction occurs between phenyl and *p*-bromophenyl *iso*cyanates and the free amino-groups of the protein molecule to form phenylureido-protein compounds, and it is difficult from the purely chemical aspect to obtain more definite information about this reaction. It seems probable that the most satisfactory evidence will be that obtained as a result of the application of immunological methods. The immunological results will be discussed more fully in the second part of this paper, but it might be mentioned here that they indicate that a chemical alteration in the protein molecule takes place as a result of the action of phenyl and *p*-bromophenyl *iso*cyanates and that the reaction involves the free amino-groups of the lysine molecules of the protein.

From the chemical point of view, further studies are being made of the phenylureido- and *p*-bromophenylureido-protein compounds. They are readily soluble in water if sufficient NaOH solution is added to give p_H about 7·5, and they are precipitated from solution at about p_H 4 to 4·5. The *p*-bromophenylureido-compounds of caseinogen and gelatin have been precipitated with alcohol *plus* acetic acid, washed with alcohol, then with an alcohol-ether mixture and finally extracted with ether for 200 hours. The products obtained were stable white preparations which were soluble at p_H 7·5, with the application of a little heat (up to 35°) in the case of the gelatin compound, and from these solutions the protein could be reprecipitated again with acetic acid. Phenylureido-gelatin and *p*-bromophenylureido-gelatin readily form gels.

II. IMMUNOLOGICAL INVESTIGATIONS.

Immunisation. The phenylureido-compound of horse serum-globulin was prepared as described in the first part of this paper and the solution kept in the ice-chest. A short time before it was required for injection purposes, phenol was added to give a concentration of 0·25 % in the solution.

This solution was injected into 5 rabbits at weekly intervals, most of the injections being intraperitoneal, although a few of the earlier injections were made intravenously. The amount of solution injected into each rabbit at each injection was such as to contain approximately 0·25 g. of the protein. The sera of the rabbits were tested 7 days after the third and subsequent injections. Three rabbits gave good antisera after the third injection, whereas the other 2 rabbits required 4 injections.

Precipitin tests. Two drops of the immune serum were added to 0·25 cc. of the diluted antigen solution in a small test-tube (2″ × ¼″). The mixture was shaken and observed after it had been incubated at 37° for ½ hr., 1 hr. and 2 hrs., and usually after a further 18 hrs. at room temperature (15–20°). Unless otherwise stated, the readings given in the Tables are those obtained after 1 hr. at 37°.

The various concentrations of antigen tested (1 : 20, 1 : 100, 1 : 500 and 1 : 2500) correspond to dilutions of a 5 % protein solution, *i.e.* the solutions used contained 0·25, 0·05, 0·01 and 0·002 % of protein respectively.

The extent of precipitin formation is recorded as follows (in increasing degrees of precipitation):

– (no reaction), f.tr. (faint trace), tr. (trace), ±, +, +±, ++, *etc.*

Preparation of phenylureido-amino-acid compounds.

These compounds were prepared by the action at room temperature of phenyl *iso*cyanate (1·3 mol.) on a solution of the amino-acid (1 mol.) containing NaOH (1 mol.). The products were separated by the addition of HCl and were recrystallised from hot water. The glycine and alanine compounds gave melting-points similar to those recorded by Paal [1894].

Preparation of phenylureido-compounds of chicken and rabbit serum-proteins.

These preparations were similar to those of the corresponding compound of horse serum-globulin described in the first part of this paper. The quantities used were 10 cc. of serum, 10 cc. of phosphate buffer (p_H 8·0), 1 cc. of 10 % NaCl and 0·4 cc. of phenyl *iso*cyanate.

Experimental.

The sera obtained by the injection of phenylureido-globulin (horse serum) into rabbits were tested against horse serum (and the separated horse serum-globulin) and against phenylureido-horse serum-globulin. Very powerful reactions were obtained with the last named antigen, indicating the possession of good antigenic power by the phenylureido-protein compound. With horse serum, or the separated horse serum-globulins, three of the antisera gave moderate and the other two very feeble or no reaction (*cf.* Table II).

These findings appear to indicate the loss, to some extent at least, of the original species-characteristics, especially since good reactions were obtained between antisera to phenylureido-horse serum-globulin and the phenylureido-compounds of chicken serum-proteins and similar compounds of rabbit serum-proteins; no reaction was obtained, of course, with untreated chicken or rabbit serum. Antisera to horse serum (or horse serum-globulin) gave good precipitin reactions with phenylureido-horse serum-globulin, so that in this respect the "horse" characteristics of the globulin have not been entirely lost as a result of the action of phenyl *iso*cyanate.

Further tests were made to determine whether these antisera to the phenyl *iso*cyanate compounds of horse serum-globulin reacted with other phenylureido-

Table II. *Precipitin reactions between anti-sera to phenylureido-horse serum-globulin and phenylureido-compounds of various serum proteins.*

Antigen	Dilution of antigen	Immune serum against					
		Phenyl *iso*cyanate compounds of horse serum-globulin				Horse serum	Horse serum-globulin
		No. 51	No. 52	No. 53	No. 54	No. 145	No. 43
Horse serum	1 : 20	±	tr.	–	–	+ +	+ + ±
	1 : 100	+ ±	+	–	–	+ +	+ +
	1 : 500	+	tr.	tr.	–	+	+
	1 : 2500	tr.	tr.	f.tr.	f.tr.	tr.	tr.
Horse serum-globulin	1 : 20	+	tr.	–	–	+ + ±	+ + +
	1 : 100	+ ±	+	f.tr.	–	+ +	+ +
	1 : 500	±	±	±	f.tr.	+	+
	1 : 2500	tr.	f.tr.	f.tr.	–	f.tr.	tr.
Phenyl *iso*cyanate of horse serum-globulin	1 : 20	+ + +	+ + +	+ + ±	+ +	+ + ±	+ + +
	1 : 100	+ +	+ +	+ ±	+ +	+ +	+ +
	1 : 500	+ ±	+ ±	+ ±	+ ±	±	+
	1 : 2500	+	+	+	+	tr.	tr.
Phenyl *iso*cyanate of chicken serum-proteins	1 : 20	+	+ +	+	+	–	–
	1 : 100	+ ±	+ ±	+ ±	+	–	–
	1 : 500	+	+ +	+	+	–	–
	1 : 2500	±	±	±	±	–	–
Phenyl *iso*cyanate of rabbit serum-proteins	1 : 20	+	+	±	+	–	–
	1 : 100	+	+ ±	+	+	–	–
	1 : 500	+ ±	+ ±	±	+	–	–
	1 : 2500	±	±	±	±	–	–

Note. The results with serum No. 50 are not included in the above Table since this serum and serum No. 51 gave practically identical results.

protein compounds, those of caseinogen and gelatin (*cf.* Tables III and IV). The results show that all these antisera gave good precipitin reactions with phenylureido- and *p*-bromophenylureido-caseinogen and also with similar compounds of gelatin, the results with the gelatin compounds being of special interest

Table III. *Precipitin reactions with various caseinogen compounds.*

Antigen	Dilution of antigen	Immune serum against											
		Phenyl *iso*cyanate compounds of horse serum-globulin								Horse serum		Horse serum-globulin	
		No. 50		No. 52		No. 53		No. 54		No. 145		No. 43	
Phenyl *iso*cyanate compound of chicken serum-proteins	1:20	±	±	+ ±	+ +	±	±	+	+	–	tr.	f.tr.	f.tr.
	1:100	+	+	+ ±	+ +	+	+	+	+	–	–	f.tr.	f.tr.
	1:500	+	+	+ ±	+ ±	±	+	+	+	–	–	–	–
	1:2500	±	±	±	+	±	±	±	±	–	–	–	–
Caseinogen	1:20	–	–	–	–	–	–	–	–	–	–	–	–
	1:100	–	–	–	–	–	–	–	–	–	–	–	f.tr.
	1:500	–	–	–	–	–	–	–	–	–	–	–	f.tr.
	1:2500	–	–	–	–	–	–	–	–	–	–	–	–
Caseinogen-phenyl *iso*cyanate compound	1:20	±	±	±	+	tr.	±	tr.	±	–	–	–	–
	1:100	+	+	+	+ ±	±	+	±	+	–	–	–	–
	1:500	+	+	+ ±	+ ±	+	+	+	+	–	–	–	–
	1:2500	+	+	+	+ ±	+	+	+	+ ±	–	–	–	–
Caseinogen-*p*-bromo-phenyl *iso*cyanate compound	1:20	±	±	±	±	–	f.tr.	±	±	–	–	–	–
	1:100	±	+	+	+ ±	tr.	±	±	±	–	f.tr.	f.tr.	f.tr.
	1:500	+	+	+	+ ±	±	+	+	+	–	–	f.tr.	f.tr.
	1:2500	tr.	±	tr.	±	±	±	±	±	–	–	–	–

Note. In this table, and in Table IV, the first reading is that obtained after the tubes had been incubated at 37° for half an hour, and the second reading is that made after one hour at 37°.

Table IV. *Precipitin reactions with phenylureido-gelatin compounds.*

Antigen	Dilution of antigen	Immune serum against											
		Phenyl *iso*cyanate compounds of horse serum-globulin								Horse serum-globulin		Horse serum	
		No. 51		No. 52		No. 53		No. 54		No. 43		No. 145	
Phenyl *iso*cyanate compound of chicken serum-proteins	1:20	+±	+±	++	++	+	+±	+	+	–	f.tr.	f.tr.	tr.
	1:100	+	+	+±	+±	+	+±	+	+	–	f.tr.	–	–
	1:500	+	+	+±	+±	±	+	+	+	–	–	–	–
	1:2500	+	+	+	+	+	+	+	+	–	–	–	–
Gelatin	1:20	–	–	–	–	–	–	–	–	–	–	–	–
	1:100	–	–	–	–	–	–	–	–	–	–	–	–
	1:500	–	–	–	–	–	–	–	–	–	–	–	–
	1:2500	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin-phenyl *iso*cyanate compound	1:20	–	tr.	–	tr.	–	tr.	–	tr.	–	–	–	–
	1:100	–	±	f.tr.	±	–	f.tr.	tr.	f.tr.	–	–	–	–
	1:500	±	+	+±	+	±	±	+	+	–	–	–	–
	1:2500	+	+	+	+	+±	+	+±	+	–	–	–	–
Gelatin-*p*-bromo-phenyl *iso*cyanate compound	1:20	–	–	–	–	–	–	f.tr.	–	–	–	–	–
	1:100	tr.	f.tr.	tr.	±	tr.	tr.	tr.	tr.	–	–	–	–
	1:500	±	±	+	+	±	±	±	±	–	–	–	–
	1:2500	+	+	+	+	+	+	+	+	–	–	–	–

in view of the fact that gelatin itself is non-antigenic. The results given in these two Tables extend, therefore, the observations with the phenylureido-compounds of various serum-proteins, and they show that there exists a close immunological relationship between the phenylureido-compounds of serum-proteins and those of such widely different proteins as gelatin and caseinogen.

Finally, in order to obtain, if possible, confirmation of the view that the reaction between phenyl *iso*cyanate and proteins involves the free amino-groups of the protein molecule and to attempt to define more precisely the nature of the grouping responsible for the new immunological specificity, a few preliminary inhibition tests have been carried out. Phenylureido-compounds of glycine, alanine and lysine were prepared and the influence of these compounds and the untreated amino-acids on various precipitin reactions determined.

Table V. *Inhibition tests.*

0·20 cc. of *M*/100 solution of the "inhibiting" substance, *plus* 0·05 cc. of the antigen solution (1 : 20 or 1 : 100), *plus* 0·10 cc. of the antiserum.

Antigen	Antiserum against	Antigen dilution	"Inhibiting" substance						
							Phenylureido-compounds of		
			NaCl	Glycine	Alanine	Lysine	Glycine	Alanine	Lysine
Horse serum	Horse serum	1:20	++	++	++	++	++	++	++
		1:100	+±	+±	+±	+±	+±	+±	+±
Phenylureido-chicken serum-proteins	Phenylureido-horse serum-globulin	1:20	+±	+±	+±	+±	±	±	–
		1:100	+±	+±	+±	+±	tr.	tr.	–
Horse serum	Phenylureido-horse serum-globulin	1:20	+	+	+	+	+	+	+

The results, given in Table V, show that all these phenylureido-amino-acid compounds inhibit to a very marked extent, in the case of the lysine compound completely, the reaction between phenylureido-proteins and the corresponding antiserum, whereas these substances have no influence at all on the formation of precipitates in other antigen-antibody reactions (*e.g.* the precipitin reaction between horse serum-proteins and an immune serum against horse serum-proteins). In the experiments recorded in Table V, the phenylureido-amino-acid compounds and the amino-acids were used in equimolecular amounts, but since there appeared to be a possibility that the greater influence of the phenylureido-lysine compound compared with the corresponding glycine and alanine derivatives might be due to the presence in the lysine compound of two phenylureido-groupings, similar tests were always made in addition with one-half that molecular concentration of the lysine compound. The smaller amount of phenylureido-lysine invariably gave complete inhibition of the "phenylureido" precipitin reaction.

DISCUSSION.

The immunological investigations described here were mainly directed to the study of the effect of treatment with phenyl *iso*cyanate on the antigenic power and the specificity of certain protein antigens. In brief, the results of these studies (Tables II, III and IV) show that treatment with phenyl *iso*cyanate does not lead to any significant loss of the antigenic power of serum-globulin. but it does lead to a marked change in specificity. Thus, antibodies to the altered protein (phenylureido-horse serum-globulin) will react very well with the antigen used for the injection and with any similarly altered protein (phenylureido-chicken serum-proteins, phenylureido-caseinogen, phenylureido-gelatin, *etc.*) and to a moderate or very slight extent only with the protein which was used for the preparation of the phenylureido-protein injected (in this instance, horse serum-globulin). This chemical treatment has resulted, therefore, in a very pronounced alteration in immunological specificity, the old species-specificity being replaced by a specificity characteristic for the new type of protein used. That the old species-specificity is not completely lost, however, is shown by the fact that some of the antisera to the phenyl *iso*cyanate-protein compounds react with the unchanged homologous proteins, and by the good reaction between these phenyl *iso*cyanate-protein compounds (phenylureido-horse serum-globulin) and antisera to the unchanged protein (horse serum-globulin). This retention of some of the original species-specificity appears to indicate the presence in the altered protein molecule of some or all of those groupings or structures which normally determine species-specificity, and this might be due (*a*) to the fact that these groups are not acted upon or interfered with in any way by phenyl *iso*cyanate, or (*b*) to an incomplete reaction between the protein and phenyl *iso*cyanate. The second possibility has certainly a fair amount of experimental support, since the amino-N determinations (Table I) show that with the proteins used for the injections (horse serum-globulin and its phenylureido-derivative), the phenylureido-compound still retains about one-third of its free amino-N, from which it might be concluded that only two-thirds of the reactive groups of the protein molecule had combined with phenyl *iso*cyanate. Without further evidence, however, it would not be justifiable to conclude that the groups of the protein molecule which react with phenyl *iso*cyanate are concerned with the determination of species-specificity, but the evidence presented here seems to offer some support in favour of such a suggestion. It would be difficult, otherwise, to explain why some antisera to phenylureido-horse serum-globulin give a very faint or a negative reaction with unchanged horse serum-globulin, and the most reasonable conclusion is that the immunologically significant groups in the horse serum-globulin have been altered or destroyed by the phenyl *iso*cyanate. A possible alternative explanation, however, would be that the introduction of any new grouping into a protein molecule produces a new structural arrangement which is more prominent than are the other groupings of the molecule in determining antibody formation when the new protein is injected into an animal. Thus the injection of phenylureido-horse serum-globulin would, according to this view, produce antibodies which are mainly directed against the phenylureido-grouping and would occasion no significant antibody response to the groupings which determine the specificity of horse serum-globulin, although these latter groupings may be unaltered by the treatment with phenyl *iso*cyanate.

Whichever explanation is adopted, however, and the weight of evidence appears to be in favour of the view that the groups acted upon by phenyl *iso*cyanate are concerned in part at least with the original species-specificity, it is of importance to know the nature of the chemical reaction between phenyl *iso*cyanate and the protein molecule. From the evidence produced in the first part of this paper, principally relating to the diminution in free amino-N and the relationship between this reduction and the amount of bromine in the *p*-bromophenyl *iso*cyanate-protein compounds, the conclusion is reached that phenyl *iso*cyanate reacts with the free amino-groups of the protein molecule. The free amino-N of proteins represents, according to Van Slyke and Birchard [1913] and later authors, one-half of the lysine nitrogen, indicating that one amino-group only of the lysine molecule is bound. This would suggest that with phenyl *iso*cyanate the reaction takes place with the free amino-groups of the lysine, presumably the ϵ-amino groups, and from the chemical evidence presented here there is much support for this view. In addition to the evidence presented in the first part of this paper, it might be mentioned that attempts to prepare *p*-bromophenyl *iso*cyanate compounds of zein, a protein deficient in lysine, have been unsuccessful. Furthermore, the bromine contents of the *p*-bromophenyl *iso*cyanate compounds of caseinogen and gelatin usually represent 70 to 90 % of the theoretical values calculated from the figures given in the literature for the lysine contents of caseinogen and gelatin. The chemical evidence although suggestive is not conclusive, however, and it is left for serological inhibition tests to supply further support for the view that phenyl *iso*cyanate reacts with the free amino-groups of the lysine molecules.

Landsteiner [1920] showed that simple organic substances which could be coupled with proteins, although they showed no antigenic power themselves, were able to combine with the specific antibodies against the coupled proteins and could in this way prevent the formation of a precipitate when the coupled protein was added. Thus *m*-aminobenzoic acid added to an immune serum against *m*-aminobenzoic acid-azo-protein prevents the formation of a precipitate when *m*-aminobenzoic acid-azo-protein is added. These inhibition tests have since been used with success in Landsteiner's laboratory [Landsteiner and van der Scheer, 1928; 1931; 1932; Wormall, 1930] and by other authors [Klopstock and Selter, 1928; Avery and Goebel, 1929] and all the evidence available points to a marked specificity of this test. It is possible by means of this test to demonstrate inhibition by a substance with configuration identical with or very similar to that of the grouping attached to the protein molecule (Landsteiner; Landsteiner and van der Scheer) or it is possible to demonstrate the presence in a chemically altered protein of certain specific groupings, such as the 3 : 5-dihalogenated tyrosine groupings in iodinated and brominated proteins (Wormall). These inhibition tests have proved extremely useful in many of the investigations already quoted and it appears probable that they will in the future be of considerable assistance in studies on the chemistry of proteins by immunological methods. It is not proposed to discuss here at any length the significance of inhibition tests with the phenyl *iso*cyanate-protein precipitin reaction, but it will perhaps be sufficient to say that such tests have served to confirm the view that the immunological specificity of these new protein compounds is due to the phenylureido-amino-acid groupings, in all probability the phenylureido-lysine grouping. As the preliminary results in Table V indicate, the precipitin reaction between phenylureido-proteins and antibodies to these protein compounds is not inhibited by certain amino-acids but is strongly inhibited by the corresponding phenylureido-amino-acid compounds, phenylureido-lysine being more effective than the corresponding glycine and alanine compounds. Other phenylureido-amino-acid compounds have still to be tested, but it appears justifiable to conclude from these early tests that the phenyl *iso*cyanate-protein compounds contain phenylureido-lysine groupings. Since this inhibition is so remarkably specific, and since phenylureido-lysine effects complete inhibition, it seems probable that no significant change, other than the formation of phenylureido-groupings, occurs when phenyl *iso*cyanate acts on proteins. If any other groups were introduced, or if any other significant change was effected in the protein molecule, the inhibition with phenylureido-lysine would probably be incomplete, since this compound has no inhibitory influence on the formation of other precipitates, such as that between horse serum-proteins and antisera to the phenyl *iso*cyanate compounds of horse serum-globulin, a reaction due to the retention in these phenyl *iso*cyanate-protein compounds of some of the horse specificity characteristics.

These immunological studies appear to suggest, therefore, that the lysine molecules of the protein are of some significance in determining species-specificity, and these conclusions would fall into line with those of Landsteiner and his co-workers, who have shown that changes which are not related to changes in the aromatic groupings of the protein molecule may destroy the species-specificity and may produce a specificity associated with the new group introduced; for example, acetylation, esterification and methylation result in a new specificity characteristic for the type of altered protein used [*cf.* reviews by Hartley, 1931; Landsteiner, 1927; 1933; Wells, 1929]. Formaldehyde has a somewhat different effect, since it converts rabbit serum-proteins into compounds which will produce in the rabbit antibodies to the antigen injected but not to formaldehyde-treated proteins of any other species [Landsteiner and Jablons, 1914]. There is perhaps sufficient evidence that the aromatic groupings of the protein are concerned in some way with specificity (for a review of the literature *cf.* Wormall [1930]), and yet there is quite as much or even more support for the view that non-aromatic groupings determine species-specificity. It may be that the introduction of any new grouping, if the group is sufficiently prominent, will lead to the acquirement of a new immunological specificity characteristic for the group introduced. With reference to the question of the significance of lysine, it is of interest to note that for a protein to be fully antigenic it is not necessary for it to contain lysine, since zein and gliadin contain no significant amount of lysine, but have marked antigenic powers. Thus all types of protein specificity cannot be related to the lysine groups.

The results of the experiments carried out with the phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate compounds of gelatin (Table IV) show that these compounds readily give precipitates with antisera to phenylureido-horse serum-globulin. This appears to be of special interest in view of the non-antigenicity of gelatin, and further investigations are being carried out to determine whether the introduction of aromatic groups into the gelatin molecule in this manner leads to the development of full antigenic power. Phenylureido- and *p*-bromophenylureido-gelatin compounds are being injected into rabbits, and tests are being made to determine whether antibody production occurs. The results of these experiments will be reported later. Adant [1930] has succeeded in obtaining precipitin reactions with suitable antisera and gelatin coupled with diazotised aniline, and Medveczky and Uhrovits [1931] have obtained similar results with benzoylated gelatin. Adant reports that the gelatin-diazotised aniline complex is fully antigenic.

The results of the immunological tests with the *p*-bromophenylureido-proteins are almost identical with those with the phenylureido-compounds, and antibodies to the latter readily react with the former, although the intensity of the reaction may not always be quite so strong. This result is perhaps what one would expect, the introduction of a bromine atom effecting a difference in the new grouping which does not alter the specificity but reduces somewhat the intensity of the reaction. These observations support, therefore, the conclusions of Landsteiner and van der Scheer [1927], who found that the specificity of azo-proteins is not necessarily greatly modified by the introduction into the aromatic nucleus of the diazo-compound of various groups, such as CH_3, NO_2, Br, Cl, *etc.*

In conclusion, it appears from the results already obtained with the phenyl *iso*cyanate-protein compounds used in the work described here that this type of compound will be of some considerable use in the study of the protein molecule from the immunological point of view. For this purpose, this method of altering the molecule or introducing new groupings has certain very definite advantages. It is relatively rapid and is not very drastic, since it can be carried out at low temperatures and in neutral or practically neutral solution. Furthermore, the excess of the reagent reacts with water to give an insoluble substance which can readily be removed.

SUMMARY.

1. Phenylureido- and *p*-bromophenylureido-compounds of caseinogen, gelatin and horse serum-globulin have been prepared by the action of phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate on solutions of these proteins kept at p_H 7 to 8. The properties of these compounds are described.

2. Evidence is presented which suggests that phenyl *iso*cyanate and *p*-bromophenyl *iso*cyanate react only with the free amino-groups of the lysine molecules of the protein.

3. The phenylureido-derivative of horse serum-globulin, when injected into rabbits, produces antibodies which give precipitin reactions with the phenylureido- and *p*-bromophenylureido-compounds of horse serum-, chicken serum- or even rabbit serum-proteins, and with similar compounds of caseinogen and gelatin. Thus, antibodies capable of reacting with any phenylureido-protein are produced.

4. The antisera to phenylureido-horse serum-globulin do not react very strongly with the unchanged globulin; indeed, with one immune serum of this type, no precipitate at all was given with horse serum-globulin. Thus there appears to be a considerable loss, when the serum-proteins are converted into the corresponding phenylureido-compounds, of the original species-specificity or characteristics, although antisera to native horse serum-globulin give very marked precipitates with phenylureido-horse serum-globulin. The significance of these findings is discussed.

5. The precipitin reaction between phenylureido-proteins and the antisera to these compounds is specifically inhibited by phenylureido-amino-acid compounds (glycine, alanine, and lysine). Inhibition is complete with the lysine

compound and not quite complete with the other phenylureido-compounds tested. This supports the view that the characteristic grouping in the new phenylureido-proteins is phenylureido-lysine.

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XCVIII. THE RÔLE OF TRYPTOPHAN IN BLOOD DEVELOPMENT.

By ROBERT SAXELBY ALCOCK[1].

From the Biochemical Laboratory, Cambridge.

(*Received April 3rd, 1933.*)

The importance of a complete knowledge of the nature of the various types of anaemia makes a settlement of the question of the precursors of the blood pigments desirable; indeed, the work of Fontés and Thivolle [1930] suggests that imperfect assimilation of certain amino-acids is the basis of at least some forms of anaemia.

This work was carried out on rabbits and dogs, in both cases without any previous treatment to reduce the haemoglobin content of the blood. A solution of tryptophan and histidine, in the proportion calculated from their supposed fates in the synthesis of the haematin and globin portions of haemoglobin respectively, was injected into the animals, and the changes in the red blood cell count, and in the haemoglobin percentages, were followed. In all cases there resulted from the injections increases in both figures.

Fontés and Thivolle next experimented to see whether lack of tryptophan in the diet would produce anaemia. Groups of rats were kept on a tryptophan-low diet, and after 10 weeks they were found to be anaemic. Finally these workers injected subcutaneously their tryptophan-histidine mixture into patients suffering from various forms of anaemia. In some cases cures were effected.

Later, Okagawa and Tatsui [1931], showed that animals recovering from anaemia, caused either by haemorrhage or by phenylhydrazine poisoning, recovered more rapidly when given injections of tryptophan. It was also found [Matsuoka and Nakao, 1931] that 2-methyltryptophan is effective in diminishing the time required for recovery from anaemia.

The results obtained by the injection of tryptophan are open to at least two explanations. One is that given by the above workers, namely that the tryptophan exerts its influence by actually forming part of the building material for the new blood. The other is that tryptophan has a stimulating influence on the blood-forming organs without necessarily being used up in the process.

The work of Fontés and Thivolle with rats, however, seems to suggest that the tryptophan does play more the part of building material than stimulator, and it is to be regretted, on this account, that these workers did not control their diets by similar ones to which tryptophan had been added. It seems likely that, had they done so, the results would have appeared less clear-cut, for the diet they were giving was inadequate in many respects.

Before making any study of the tryptophan metabolism of anaemics it seemed desirable to attempt to settle this fundamental question of the rôle of tryptophan with regard to blood formation.

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[1] 1851 Senior Student.

Experiments have been carried out to determine whether the lack of tryptophan, and of tryptophan alone, does really produce anaemia, as Fontès and Thivolle state, and whether rats already anaemic can recover from their anaemia while on a diet lacking in tryptophan.

Preliminary experiments were also performed to see whether rats would respond to tryptophan and histidine injections, but it was not possible to show any effect. Rabbits similarly failed to respond.

EXPERIMENTAL.

Tryptophan-free diet.

The diet employed by Fontès and Thivolle in their experiments on rats is unsatisfactory in having gelatin as its sole protein. Rats do not digest gelatin well, and even predigested gelatin supplemented with the missing amino-acids does not give good growth.

Acid-hydrolysed caseinogen as sole protein failed to give satisfactory growth, nor did the animals thrive on a diet of which the protein consisted of zein supplemented with whole or acid-hydrolysed gelatin. The protein which has proved most successful is a mixture of equal parts of acid-hydrolysed caseinogen and zein. The last series of experiments was carried out, however, using only acid-hydrolysed caseinogen, as there is some possibility that all samples of zein contain some tryptophan.

The vitamins were given in the form of three drops of cod-liver oil per rat, and 0·75 cc. of an alcoholic extract of marmite, corresponding to 0·75 g. yeast per rat daily. This supplement provided the rats with less than 1 mg. of tryptophan daily.

After an initial period of rapid fall, there is little loss of weight on the tryptophan-free diet described. The animals get into low bodily condition and are liable to ulcers, but it has been possible to keep them alive for 5 months without tryptophan.

The composition of the tryptophan-free diet was as follows:

	%
Acid-hydrolysed caseinogen (autoclave) ...	9
Zein	9
Rice starch	40
Palm kernel oil	17
Sucrose	20
Salt mixture	5

The dry food was mixed with water to form a stiff paste, and was administered *ad lib.* The animals ate 6–8 g. daily.

The animals used were of a stock having red blood cell counts between 8 and 9 millions per mm.^{3}, and haemoglobin values between 80 and 90 %. Blood was drawn by snipping a small piece off the end of the tail, and as it was expected that weekly or fortnightly readings would be necessary, not all the values were determined at the beginning, and in these cases the above limits may be accepted.

There was a loss of weight of between 20 and 50 % of the original, but this bore no relationship to the blood picture or even to the duration of the experimental period.

The average final values (Table I) are, erythrocyte count, 8·7 mm.; haemoglobin value, 84 %.

Table I.

Rat	Sex	Duration of experiment days	Initial R.B.C. count millions/mm.^{3}	Final R.B.C. count millions/mm.^{3}	Final Hb %
9	F	40	9·7	9·4	—
10	M	40	7·8	8·2	—
11	M	40	9·1	9·3	—
30	F	52	—	8·9	80
12	F	60	—	6·6	74
13	F	60	—	10·5	90
8	F	100	8·5	6·8	80
21	F	100	—	9·5	100
22	F	110	—	8·9	82

Of the above animals, only Nos. 8 and 12 were in any way below the normal, and in both cases there had been ulcers on the tail, while the animals were in very poor bodily condition. In any case, the diminution in the haemoglobin figure for rat 12 is in no way comparable with that occurring in milk anaemia.

Otherwise, the blood figures were normal, in direct contrast with the finding of Fontés and Thivolle.

In the second series of experiments, the rats were made anaemic by the technique of Elvehjem and Kemmerer [1931], and 3–4 weeks after weaning the erythrocyte counts lay in the region of 2 millions per mm.^{3}, and the haemoglobin percentage round 30.

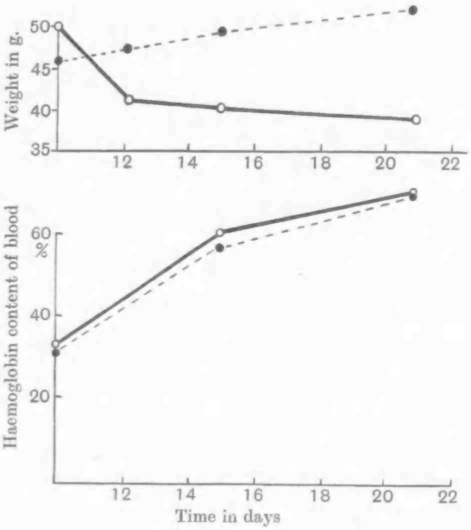

Fig. 1. Continuous lines, deficient group 2. Broken lines, control group 2.

Four rats made anaemic in this way were divided into two groups of 2, the first being given the tryptophan-deficient diet without supplement, the latter, the same, with 0·5 % of tryptophan added. The red blood corpuscle counts were taken from the outset and the haemoglobin percentages in the later stages.

The deficient animals failed to put on any weight, and one succumbed at the end of 4 days. The survivor lived 17 days during which the erythrocyte count rose from 2·0 to 6·0 millions. The haemoglobin percentage at this stage was 90.

The animals receiving the supplement gained in weight, and the blood figures were nearly normal at the end of 3 weeks. The results are recorded in Table II.

Table II.

Group	No. of rats	Time in days	Weight		R.B.C. count millions/mm.^{3}		Haemoglobin %	
			Initial	Final	Initial	Final	Initial	Final
Deficient 1	1	17	52	44	2·0	6·0	—	90
Control 1	2	17	66	96	2·5	5·6	—	90
Deficient 2	5	11	50	40	—	—	32·7	69·2
Control 2	4	11	46	51	—	—	31·7	68·5

The experiment was repeated using 6 rats in the deficient group and 4 in the supplemented. Of the former, 5 survived for a fortnight—long enough for recovery from anaemia. Haemoglobin percentages alone were recorded in this case, and the results are shown in Table II and in Fig. 1. It is clear that the lack of tryptophan in the diet is having little influence upon the recovery from anaemia in these cases, and the very poor bodily condition of the animals almost precludes the possibility of drawing on reserves for the necessary building materials.

Discussion.

The failure of tryptophan-deficient animals to develop anaemia, while not sufficient in itself to prove that blood haematin is not a tryptophan product, is more readily explained if this is the case. The loss of weight over the experimental period is so slight, in the later stages, as to demand an extraordinary economy of endogenous tryptophan if this is indeed the source of pyrrole-nitrogen throughout the months of dietary lack of tryptophan.

The recovery from anaemia of tryptophan-starved animals is difficult to fit in with the idea of the utilisation of this amino-acid in haematin building. The exact parallel in the recovery in the two groups of animals in these experiments makes it almost certain that haematin-pyrrole is not derived from tryptophan.

It remains to explain the results of the other workers. The anaemia developed by the rats used by Fontés and Thivolle may well have been due to deficiency of some other dietary constituent besides tryptophan. The results of work involving injections can be explained on the basis of a stimulating action of tryptophan on the blood-forming organs. Alimentary tryptophan could not be expected to exert such an effect, as excess of this amino-acid in the portal system is converted into kynurenic acid in the liver. If this hormone-like effect is real, it might be expected that compounds similar to tryptophan would exert a similar action. Matsuoka and Nakao [1931] were able to show that 2-methyltryptophan, while not able to replace tryptophan in the diet, will nevertheless accelerate the recovery from phenylhydrazine-induced anaemia, when injected.

Summary.

Rats kept on a tryptophan-deficient diet over prolonged periods failed to develop anaemia.

Young rats suffering from milk anaemia recovered from this anaemia independently of the presence of tryptophan in their diet.

I should like to take this opportunity of expressing my indebtedness to Sir F. G. Hopkins, for his encouragement and advice.

Part of this work was carried out during the tenure of a grant from the Department of Scientific and Industrial Research.

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XCIX. THE ANAEROBIC DECOMPOSITION OF *l*-CYSTINE BY WASHED CELLS OF *PROTEUS VULGARIS*.

By HUGH LEWIS AUBREY TARR[1].

From the Biochemical Laboratory, Cambridge.

(*Received April 29th, 1933.*)

Adequate summaries of the literature dealing with the products resulting from the decomposition of amino-acids by various bacteria have been prepared by Stephenson [1930] and Buchanan and Fulmer [1930]. In the case of cystine a study of these reviews and of more recent literature makes it evident that previous investigators have concerned themselves with the formation of hydrogen sulphide, ethylmercaptan and diethyl sulphide from cystine, and not with the complete disintegration of the molecule. In the present paper the anaerobic decomposition of cystine by means of "washed cells" [Sandiford and Wooldridge, 1931] of *Proteus vulgaris* is fully described.

Experimental.

The strain of *Proteus vulgaris* employed was obtained from the Department of Pathology, Cambridge. The organism was inoculated in the form of a thin suspension on to the surface of caseinogen digest agar [Cole and Onslow, 1916] sloped in 1 litre Roux bottles. After 24 hours at 37° the organisms were washed from the surface with a sterile sulphate solution (Na_2SO_4, 0·9 %; K_2SO_4, 0·05 %), centrifuged under aseptic conditions and washed once with the same solution. Subsequently the bacterial cells were suspended in sterile sulphate solution to give a fairly thick suspension, the dry weight of bacteria being reported for each experiment. Such suspensions were stored at 1° under aseptic conditions and were used within 5 days of preparation. A sulphate solution was employed in the place of Ringer's solution since the presence of chloride interfered with subsequent volatile acid determinations.

The cystine employed gave the following analytical figures: N, 11·34; S, 26·24 %; theoretical N, 11·67; S, 26·69 %.

Fig. 1 shows the aeration train employed in the experimental work. The flasks employed had the following capacity: *A*, 2000 cc.; *B*, 500 cc. and *C*, 150 cc. The technique was as follows. Exactly 500 mg. of cystine were weighed out and placed in flask *A*, the rubber stopper and glass tubing inserted as illustrated, and the whole sterilised by autoclaving for 20 minutes at 15 lbs. pressure. The cystine was sterilised in the dry form since there are indications that there is a greater tendency for it to decompose when sterilised in an aqueous medium. 400 cc. of sterile $M/40$ phosphate buffer of p_H 7·8 were then added aseptically by the tube *X*, and the tube was closed by means of a screw-clip. Flask *B*, containing 300 cc. of 2 % zinc acetate solution slightly acidified by means of 1 cc. of 5 % acetic acid, was then connected as shown and a stream of hydrogen washed through 5 % $AgNO_3$, alkaline pyrogallol and 10 % H_2SO_4 bubbled rapidly through the aeration train for 2 hours in order to remove all the CO_2 present in the flasks. 100 cc. of a suspension of *P. vulgaris* were then added carefully by the tube *X*, and flask *C*, containing about 100 cc. of carbonate-free, approximate 0·1 *N* $Ba(OH)_2$, was connected to the end of the aeration train. The entire set-up was placed in an incubator at 37° and a rapid stream of washed hydrogen bubbled through the solutions for 24 hours. Experiments showed that, at the end of this time, all the cystine had dissolved and had completely broken down, the final p_H of the liquid being about 7·0 to 7·2. Nothing but typical *P. vulgaris* colonies developed on caseinogen digest agar plates prepared from suitable dilutions of the fluid in flask *A*, and smears stained by Gram's method showed nothing but negative cells which appeared to be identical with those found in the original suspensions.

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[1] Holder of 1851 Exhibition Overseas Travelling Scholarship and External Research Student Emmanuel College, Cambridge.

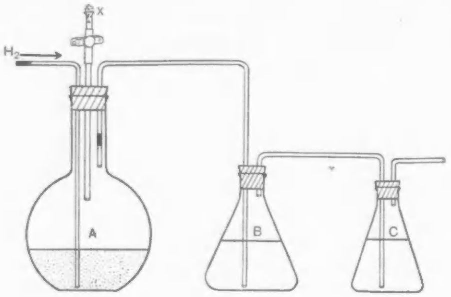

Fig. 1

Flask *A* was then placed in ice-water in order to avoid the possibility of further decomposition of any product in the solution, and, after a preliminary 15 minutes in order to allow the solution to cool, 2·5 cc. of *N* H_2SO_4 (3 cc. in control experiments) were added to bring the p_H to approximately 6·5, and hydrogen was bubbled through the solution for 2 hours in order to remove practically all remaining H_2S and CO_2. Control experiments, employing identical technique, but with no added cystine, were run under the same conditions with the same suspension in each case. The products arising from the decomposition were estimated by the following methods.

CO_2. The white $BaCO_3$ precipitate in flask *C* was filtered on a Büchner funnel and washed quickly with several successive amounts of distilled water. The CO_2 present in the precipitate was then estimated by the method described by Stephenson and Whetham [1922].

H_2S. The zinc acetate solution in flask *B* was diluted to 5000 cc., the white precipitate of zinc sulphide being thoroughly mixed in order to give a uniform suspension. 2 cc. of this suspension were taken for a colorimetric determination of H_2S by the method described by Almy [1925]. In control experiments the original zinc acetate solution was diluted to 500 cc. and 50 cc. of the resulting solution taken for the H_2S determination. The amount of H_2S formed in control experiments was negligible in comparison with that found in cystine experiments.

NH_3. The technique was based on that developed by Van Slyke [1911] for the estimation of ammonia, using the apparatus described by Stephenson and Whetham [1922] for the estimation of volatile acid. For the determinations the liquid in flask *A* was made up to exactly 500 cc. without removing the bacterial

cells. 50 cc. of the suspension were distilled with 2 g. of anhydrous Na_2CO_3 and the ammonia collected in standard H_2SO_4 under reduced pressure at a temperature not exceeding 50°.

Total volatile acid. The remaining 450 cc. of suspension from flask *A* were made alkaline to phenolphthalein with *N* NaOH, and then filtered through a layer of kieselguhr on a Büchner funnel with suction in order to remove most of the bacterial cells. The resulting clear solution was concentrated under reduced pressure to between 40 and 50 cc., and the number of cc. of volatile acid present determined by the method described by Stephenson and Whetham [1922]. The clear distillate containing the barium salts of the volatile acids was evaporated under reduced pressure and made up to exactly 100 cc. This solution, which represented 450 mg. of cystine, was employed for the estimation of formic and acetic acids.

Formic acid. The formic acid in 11·1 cc. of the above solution was estimated exactly by the technique described by Stickland [1929].

Acetic acid. The remaining 88·9 cc. of the above solution were made more strongly alkaline by the addition of 5 cc. of *N* NaOH, placed in a 150 cc. flask and heated to boiling on a wire gauze. Approximately *N*/10 $KMnO_4$ was then run into the hot solution until a purple colour resulted indicating an excess of permanganate, thus oxidising the formic acid present. The solution was cooled, and 1 % H_2O_2 added cautiously until the purple colour due to the excess permanganate disappeared. The precipitated MnO_2 was removed by filtering with suction, the clear filtrate made faintly acid with *N* H_2SO_4 and cold, saturated Ag_2SO_4 solution added in slight excess of that required to precipitate the chloride. The AgCl precipitate was removed by filtration, the solution made slightly alkaline by the addition of *N* NaOH and the resulting AgOH precipitate removed by filtration. The clear solution was evaporated under reduced pressure to between 40 and 50 cc. The amount of volatile acid present was then determined by the method already referred to with the single exception that the excess baryta in the receiving flask was titrated with standard H_2SO_4 in place of HCl. The barium sulphate was removed by centrifuging and the supernatant liquid evaporated in a large evaporating dish over a water-bath to between 25 and 30 cc. The solution was then filtered through a small filter-paper into a weighed crystallising dish. The clear solution was evaporated to dryness on a water-bath and then placed over concentrated H_2SO_4 in a vacuum desiccator for 24 hours. The dry residue was then weighed, the weight being compared with that calculated for $(C_2H_3O_2)_2Ba$, and the percentage of barium in the salt was then estimated as $BaSO_4$. A small quantity of the *para*toluidide was prepared from the mixed barium salts from two separate experiments according to the directions given by Mulliken [1904], and the melting-point of the resulting compound was determined alone and when mixed with a sample of acet*para*toluidide prepared from pure acetic acid.

The results of two typical experiments are recorded in Tables I and II. It is evident from the data obtained that cystine breaks down under the conditions of the experiments to yield two molecules each of hydrogen sulphide, ammonia, acetic and formic acids, the last named presumably partially decomposing to form H_2 and CO_2:

$$\begin{array}{l} COOH.CH(NH_2).CH_2S{-}SCH_2.CH(NH_2).COOH + H_2 + 4H_2O \rightarrow \\ \qquad\qquad 2H_2S + 2NH_3 + 2CH_3COOH + \underset{\displaystyle 2H_2 + 2CO_2}{\underset{\downarrow}{2HCOOH}} \end{array}$$

Table I. *Recovery of the various substances resulting from the anaerobic decomposition of 500 mg. of* l-*cystine.*

Exp. 1. 0·95 g. dry weight of bacteria used.
Exp. 2. 0·72 g. dry weight of bacteria used.

Experiment No.	NH_3 mg.	H_2S mg.	CO_2 mg.	Total* volatile acid in cc. of 0·1 *N*	Acetic† acid mg.	Formic acid mg.	Total‡ formic acid mg.
1							
Cystine added	74·0	139·0	114·0	60·4	250	73·5	
Control	3·4	0·2	30·8	3·1	0	0	
Difference	70·6	138·8	83·2	57·3	250	73·5	160·5
2							
Cystine added	68·0	132·0	146·0	56·9	236	80·5	
Control	3·4	0·2	43·0	3·0	0	0	
Difference	64·6	131·8	103·0	53·9	236	80·5	188·0

* The volatile acid arising in the control experiments has not been identified, and, since it represents only about 5 % of the total volatile acid formed in the presence of cystine, has been neglected.

† Barium calculated for $(C_2H_3O_2)_2Ba$ ... ... 53·79 %
Barium found, Exp. 1 ... ... 52·6 %
Barium found, Exp. 2 ... ... 52·6 %
M.P. of acet*para*toluidide, from pure acetic acid 146–7°
M.P. of acet*para*toluidide, prepared from mixed barium acetate of Exps. 1 and 2 ... ... 146–7°

No change was found in the M.P. when some of the above toluidide was mixed with *para*-toluidide prepared from pure acetic acid.

‡ The total formic acid is the sum of the formic acid found *plus* that calculated for the CO_2 recovered, assuming that formic acid breaks down according to the equation given.

Table II. *A comparison of the experimental and theoretical values based on the decomposition of cystine with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.*

	Recovery from 500 mg. cystine					
	Exp. 1		Exp. 2		Theoretical	
Compound	mg.	%	mg.	%	mg.	%
NH_3	70·6	100	64·6	92	70·2	100
H_2S	138·8	99	131·8	94	140·5	100
CH_3COOH	250·0	100	236·0	95	248·0	100
HCOOH (Total)	160·5	85	188·0	99	190·0	100

As yet all attempts to demonstrate the presence of lactic or pyruvic acid as a possible intermediary product of the reaction have proved unsuccessful. It is, therefore, impossible to state at present which of the several possible courses of anaerobic breakdown occurs.

SUMMARY.

l-Cystine has been shown to undergo anaerobic decomposition in the presence of washed cells of *P. vulgaris* with the formation of two molecules each of hydrogen sulphide, ammonia, acetic and formic acids.

I am indebted to Miss M. Stephenson and Dr E. Friedmann of this Department for advice and criticism, and to Sir F. G. Hopkins for his constant interest.

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C. POTENTIOMETRIC ANALYSIS OF PURE ANIMAL PROTEINS.

By JACQUES ERRERA and YEHUDA HIRSHBERG.
From the University of Brussels.

(*Received April 29th, 1933.*)

In previous articles one of the authors has published a series of experimental results obtained with animal and vegetable proteins by two different methods: potentiometric titration, and analysis by means of the dielectric constant [Errera, 1932, 1, 2; Errera and Bloch, 1932; Errera *et al.*, 1932].

In the experiments which form the basis of the present article, the authors had as their object the systematic study of some pure animal proteins with a view to the elucidation of the following points.

(1) The determination of the isoelectric points of these pure proteins.

(2) The stoichiometric relationship of the combinations of these proteins with acids and bases.

(3) The value of the acid and basic equivalents of these proteins.

(4) The acidic and basic dissociation constants of the proteins.

(5) The determination of the number of COOH and NH_2 groups for each of the molecules studied.

EXPERIMENTAL METHODS AND PROCEDURE.

The method of analysing a solution by potentiometric titration is well known [see *e.g.* Muller, 1932].

In order to overcome the difficulties encountered in the estimation of the p_H in the case of proteins, we have used antimony and glass electrodes.

In the previous work, only the antimony electrode was used. In the present experiments we have used either a glass electrode alone, or else we have used a glass electrode and checked the results obtained against an antimony electrode.

The use of an antimony electrode necessitates the employment of only slight precautions during the measurement; no gas is needed, and equilibrium is fairly rapidly attained in the case of protein solutions[1]. Antimony electrodes are made by melting metal of a high degree of purity in a graphite crucible under carbon monoxide. The cell in which they are to be employed is built up on the following principles: saturated calomel electrode—solution of unknown p_H—antimony electrode. The standard curve for the antimony electrode was determined with the aid of a buffer solution to which white of egg had been added. This curve consists of three straight lines joined together by very flat curves. The straight line portions of the curve may be held to correspond to three different conditions of equilibrium of the Sb in acid, neutral and alkaline media respectively.

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[1] We wish to express our thanks to Mr H. Vogels for the method of carrying out the potentiometric measurements with an antimony electrode. For the details of this method, see Vogels [1930], and also the work which Mr Vogels will shortly publish.

The glass electrode furnishes more reliable results because in this case the equilibrium is unique, and also because a little antimony always dissolves in the solution. The experimental set-up is in many respects similar to that used by one of the authors in previous experiments on the measurements of p_H in blood-plasma [Errera *et al.*, 1930]. An additional interrupter permits of working alternatively with a glass and with an antimony electrode. The concordant results obtained lead to the conclusion that neither the solution of traces of antimony nor the formation of a layer of adsorbed proteins on the surface of the glass electrode vitiates the results.

Once the results are obtained, the usual procedure is to construct a graph, using the number of cc. of the reagent added as abscissae, and the corresponding p_H values as ordinates. If however this method is adopted in the case of proteins, the resulting curves do not show the characteristic points with sufficient clearness.

Fig. 1 shows the titration curve for pig-nut globulin dissolved in NaOH. The curve shows three characteristic points of which two correspond to points of maximum inclination of slope while the third, which lies between the other two, corresponds to a point of minimum inclination. The significance of these points will be discussed later.

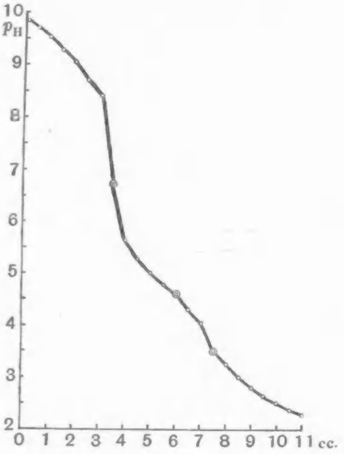

Fig. 1.

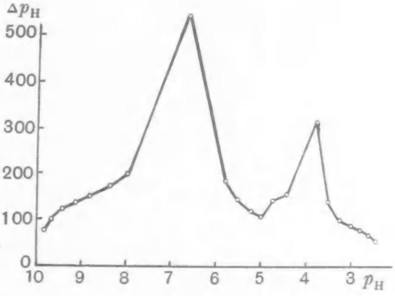

Fig. 2.

In order to bring out the experimental results more clearly, we have chosen another mode of representation: as abscissae are plotted the p_H values, and as ordinates the difference in millivolts between two[1] succeeding p_H values (Δp_H) corresponding to an addition of a constant volume of the titrating reagent. Thus for instance alkaline globulin is titrated with $N/10$ HCl, 0·5 cc. is added each time and after each measurement of the resulting p_H the difference between this and the preceding value is plotted on the graph.

Fig. 2 shows the same results as those used to construct Fig. 1, plotted according to this new method. On comparing the two curves, we see that the two maxima in Fig. 2 correspond to the two points of maximum tangential inflection in Fig. 1, while the minimum in Fig. 2 coincides with the point of minimum tangential inflection. It is at once obvious that the graph based on the differential representation brings out the characteristic points much more clearly.

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[1] The millivolt differences are calculated with respect to the final p_H.

The question now arises as to the significance of the maxima and minimum of Fig. 2. The two maxima represent the neutralisation of one component by the titrating reagent (for instance base with acid). The minimum represents the isoelectric point.

To the left of the first maximum and to the right of the second maximum the reaction proceeds independently of the protein. The first maximum represents the neutralisation of the free base and the beginning of the reaction between acid and the COONa group of the protein.

When we arrive at the isoelectric point, all the COONa groups have been converted into COOH groups by the acid, and the combination of the amino-groups of the protein with the acid now begins. At the second maximum this combination is complete, and the reaction of the free acid upon the protein begins.

In these experiments we have further used reagents of different valency: hydrochloric and sulphuric acids as acid reagents, sodium hydroxide and barium hydroxide as basic reagents. The choice of the concentration of the titrating reagent in relation to that of the protein to be titrated should only be made after carrying out preliminary investigations.

When hydrochloric acid is titrated with sodium hydroxide, a normal curve is obtained whose differential representation gives only a single maximum. If the titration is now reversed, and carried out in the presence of CO_2 from the air, two maxima are observed. This is due to the presence of $NaHCO_3$ which continues to form during the titration in the air, potentiometric titrations being extremely sensitive to small traces of this substance. This presence of bicarbonate may indeed be the cause of certain errors in the determination of the isoelectric point when this lies close to the jump in potential due to the bicarbonate. For this reason we have modified the method used in our previous experiments as follows: two portions of the isoelectric protein were titrated, the one with an acid and the other with an alkaline reagent, the correction to be made after comparison with the blank being further taken into account where necessary [see Errera, 1932, 1, 2; Errera *et al.*, 1930; 1932; Harris, 1923].

PREPARATION OF THE PROTEINS.

(*a*) *Egg-albumin.* The albumin was separated from the other proteins contained in beaten white of egg by half saturating with ammonium sulphate. The globulins thus precipitated were separated by filtration, and the albumin was precipitated by complete saturation of the filtrate with a slight excess of powdered ammonium sulphate. The albumin was then dialysed through a cellophane membrane, the water outside the membrane being changed until no reaction could be detected for the sulphate ion. Subsequently the albumin was purified by electrodialysis according to the method of Pauli and Weiss [1928] through a nitrocellulose membrane.

(*b*) *Serum-albumin.* Albumin from dog-serum was separated from the globulin by half saturating with ammonium sulphate at 37°. After filtration, the albumin was precipitated from the filtrate by complete saturation with a slight excess of ammonium sulphate, also at 37°. The purification of the albumin was subsequently carried out in the same way as for egg-albumin.

(*c*) *Haemoglobin*. This was prepared from horse blood according to the method of Svedberg and Nichols [1927] and subsequently purified by electrodialysis.

(*d*) *Caseinogen*. Very pure caseinogen prepared by the method of Hammarsten (Kahlbaum) was subsequently subjected to electrodialysis.

EXPERIMENTAL RESULTS.

(1) *Isoelectric point*.

The isoelectric point of each protein was determined by the potentiometric measurement of the p_H of the protein after purification by dialysis and electrodialysis controlled by conductivity measurements. We have found that proteins prepared in this manner are always at the isoelectric point, irrespective of whether the membrane used for electrodialysis is of nitrocellulose or of parchment.

Table I.

Protein	No of experiments	Limits of p_H of the isoelectric point	Mean of preceding column
Egg-albumin	25	4·7–4·85	4·75
Serum-albumin	15	5·4–5·5	5·45
Haemoglobin	15	6·5–6·6	6·55
Caseinogen	15	5·6–5·8	5·7

We believe that we are justified in drawing from the above results the conclusion that this method of determining the p_H of a protein purified by electrodialysis is rational and gives accurate results, at any rate in so far as the determination of the isoelectric point of simple proteins is concerned.

As we have already explained, portions of the isoelectric protein were titrated respectively with an acid and with a base. In most cases however we also titrated back: thus after having first titrated with an acid, the same solution was subsequently titrated back with a base. The curve obtained had the form shown in Fig. 2, and the isoelectric point corresponding to the minimum was thus determined again by this second method. The resulting agreement was excellent, except for small variations in certain cases, which could be traced to the interfering presence of bicarbonate.

(2) *Stoichiometric relationship of the combination of the proteins with acids and bases*.

This relationship can be found to a first approximation by means of potentiometric titration, provided care is taken not to work in a too highly alkaline medium (see previous publications). In the present experiments we have been at particular pains to establish the stoichiometric relationship starting from simple proteins purified by electrodialysis. To this end titrations were carried out on separate portions, some of which were titrated with mono- and di-basic acids, while others were titrated with a uni- and a bi-valent base. The concentration of the titrating reagent in the experiments shown in Tables II–V, varied from $N/10$ to $N/100$ according to the concentration of the protein to be titrated. In the Tables the number of cc. used have been uniformly calculated to correspond to a concentration of $N/100$. This number of cc. corresponds to the quantity of reagent fixed by the protein and is represented in Fig. 2 by the passage of the curve from the isoelectric point to one of the two maxima.

Table II.

Pure protein in water	Quantity of protein g.	Number of cc. NaOH	Same calc. for 1 g. protein	Number of cc. HCl	Same calc. for 1 g. of protein
Egg-albumin	2·7	73	27·0	15	5·5
	0·9	25	27·7	5	5·5
	0·45	13	28·9	2·5	5·5
	0·24	7	29·2	1·5	5·5
Serum-albumin	0·88	16	18·2	6·5	7·4
	0·44	8·5	19·3	3·5	7·9
	0·22	4	18·2	1·75	7·9
	0·09	1·6	17·8	0·75	8·3
Haemoglobin	0·45	7	15·6	5	11·1
	0·34	5	14·7	3·5	10·3
	0·23	3·2	14	2·5	10·8

Table III.

Protein dissolved in NaOH	Quantity of protein g.	Number of cc. HCl used between the two maxima	Same calc. for 1 g. of protein
Caseinogen	0·5	80	16
	0·2	28	14
	0·1	16	16
	0·05	8	16

Table IV.

Pure protein in water	Quantity of protein g.	Number of cc. Ba(OH)$_2$	Same calc. for 1 g. of protein
Egg-albumin	0·9	26	28·8
	0·6	17·5	29
	0·3	8	27
Haemoglobin	0·45	7·5	16·6
	0·34	5	14·7
	0·23	3·5	15·2

Table V.

Pure protein in water	Quantity of protein g.	Number of cc. H_2SO_4	Same calc. for 1 g. of protein
Egg-albumin	1·2	6·5	5·4
	0·9	4·6	5·1
	0·6	3·5	5·8

The column in which the number of cc. of reagent used is calculated for 1 g. of protein shows the stoichiometric relationship of the reaction between the protein and the acids and bases employed in terms of the concentrations of the proteins and the titrating reagent and also of the valency of the latter.

(3) Taking as a basis the above Tables, we have calculated the quantity of each protein expressed in g., which is needed to fix one g.-ion of H^+ or one g.-ion of OH^-. Table VI shows the results.

Table VI.

Protein	Gram-equivalent of protein fixing one g.-ion of OH^-	Gram-equivalent of protein fixing one g.-ion of H^+
Egg-albumin	3420	18000
Serum-albumin	5450	12900
Haemoglobin	6800	9200
Caseinogen	860	5000

(4) The fourth point which we have studied is the calculation of the dissociation constants of the pure proteins, when viewed as acids (K_a) or when viewed as bases (K_b), starting from the p_H corresponding with half-titrations (see previous publications). These values are of course only mean values, for it is probable that the different COOH and NH_2 groups do not dissociate at the same p_H, since they are situated differently in the protein molecule (compare for example the different dissociation of the various H^+ ions in the case of phosphoric acid). But the very fact that the form of the curve attained between the minimum and each of the two maxima is continuous, allows one to carry out the calculations: for it is obvious that otherwise the curve should present a series of small intermediary maxima corresponding with the various active groups.

Table VII shows the mean values obtained, together with the number of titrations on which they were based.

Table VII.

Protein	No. of titrations	Mean K_a	No. of titrations	Mean K_b
Egg-albumin	15	$3{\cdot}6 \times 10^{-7}$	10	$2{\cdot}8 \times 10^{-11}$
Serum-albumin	10	$5{\cdot}5 \times 10^{-8}$	10	$2{\cdot}7 \times 10^{-10}$
Haemoglobin	8	$1{\cdot}2 \times 10^{-8}$	6	$7{\cdot}6 \times 10^{-10}$
Caseinogen	5	$5{\cdot}0 \times 10^{-8}$	5	$3{\cdot}0 \times 10^{-10}$

(5) The fifth point is the determination of the number of COOH and NH_2 groups per molecule of protein, it being of course understood that these molecules have the dimensions of colloid particles. This calculation can be carried out if the acid and basic equivalents of the proteins (shown in Table VI) are known, and also their molecular weights. For these latter we have adopted those found by Svedberg and his collaborators.

Table VIII gives the numbers of these COOH and NH_2 groups which act as acids and bases capable of dissociation.

Table VIII.

Protein	Molecular weight	No. of COOH groups	No. of NH_2 groups
Egg-albumin	34,500	10	1·9
Serum-albumin	67,500	12·3	5·2
Haemoglobin	68,000	10	7·4
Caseinogen	98,000	114	19·6

EXPERIMENTS UNDERTAKEN AS A CHECK[1].

In order to establish the particular character of the chemical combinations of proteins, some experiments were carried out on inert adsorbent substances. Freshly precipitated $BaSO_4$, activated charcoal and silica which had been prepared by an industrial process of electro-osmosis were studied. In all three cases the potentiometric curves were similar to that obtained with pure water: and it is therefore impossible for the curves observed with proteins, except perhaps in a very small degree, to be due to adsorption phenomena.

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[1] It may be noted in passing that the experiments carried out on albumins seem to indicate that with increasing time, even in a medium of pure water, a portion of the albumin is transformed into another protein having a different and less acid isoelectric point. Further experiments are however needed.

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CI. CHEESE-RIPENING STUDIES[1].

THE NITROGEN REQUIREMENTS OF THE LACTIC ACID BACTERIA: THE EFFECT ON THE SUGAR-FERMENTING ABILITIES OF ENRICHING PEPTIC CASEINOGEN DIGEST BROTH WITH YEAST EXTRACT.

By WILFRID SADLER and BLYTHE ALFRED EAGLES.

The University of British Columbia, Vancouver, Canada.

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In studies on the quantitative determination of the sugar-fermenting abilities of lactic acid bacteria, Orla-Jensen [1919], Orla-Jensen, Orla-Jensen and Spur [1926], and Knudsen and Sørensen [1929] have shown that for certain strains—included in the genus *Betacoccus* or *Betabacterium* Orla-Jensen—the enrichment of the nitrogen source with autolysed yeast (yeast extract) assures a marked increase in the amount of acid produced from many of the carbohydrates submitted to fermentation. In view of these results, we have employed yeast extract in enriching various media used in our work on the bacteria isolated from ripening Kingston cheese [Sadler, Eagles and Pendray, unpublished results]. At the outset, milk and sugar broth respectively were enriched with yeast extract at the rate of 0·15 %—a concentration approximating to the amount used by Orla-Jensen [1919] in preparing some of his standard media.

As the study of the sugar-fermenting abilities of the bacteria progressed, we were led to enquire into the influence thereon of enriching the standard nitrogen source broth with higher concentrations of yeast extract.

EXPERIMENTAL.

Peptic caseinogen digest broth having a total nitrogen content of approximately 0·5 %—the standard broth employed by Orla-Jensen [1919]—was used as the nitrogen source. The method of preparation is given by Orla-Jensen [1919], and by Eagles and Sadler [1932]. The distribution of nitrogen in the source and also in Bacto yeast extract has been shown [Eagles and Sadler, 1932]. For the comparative studies we enriched the broth with Bacto yeast extract at the rate of 0·15, 1, 2 and 5 % respectively—an enrichment with yeast extract at the rate of 0·15 % being equivalent in terms of nitrogen to an enrichment of 0·01 % total nitrogen [Eagles and Sadler, unpublished results]. Having found that provided the "kind" of nitrogen is suitable the "amount" is equally important [Sadler, Eagles and Pendray, 1932, 1], peptic caseinogen digest broth containing approximately 1 % total nitrogen was also employed as a nitrogen source.

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The enriched broth is prepared as follows. The necessary weight of yeast extract is dissolved in a portion of the required volume of peptic caseinogen digest broth by heating at 80° for about 30 minutes—approximately 2 g. yeast extract in 100 cc. broth. The solution is then filtered, the filtrate is made up to volume with the digest broth and the whole is thoroughly mixed.

The method of preparing each sugar broth and the procedures followed throughout the fermentation study have been described already [Sadler, Eagles and Pendray, 1932, 1].

Determinations were made on 7 cultures isolated from Kingston cheese. Cultures EMB_1 173 and 195 and cultures EMB_2 168 and 173 are Gram positive, non-gelatin-liquefying coccus forms that have been already reported upon [Sadler *et al.*, 1932, 1]. Cultures EMB_1 58, EMB_2 189 and EMB 33 are Gram positive rod forms. Culture EMB_1 58 was isolated from a Kingston cheese 13 days old, culture EMB_2 189 from a cheese 2 months old, and culture EMB 33 from a cheese which, when 2 weeks old, had been placed in cold storage and held at about 37° F. for 4 months.

The results of the determinations of the total titratable acidity—recorded as g. lactic acid per litre—produced by the organisms from 7 carbohydrates, when employing the nitrogen sources defined, are given in Table I.

When employing nitrogen source 2—peptic caseinogen digest broth containing approximately 0·5 % total nitrogen [Eagles and Sadler, 1932]—the total titratable acidity produced from each of the carbohydrates by culture EMB_1 173 is closely comparable, in every case, with the total amount of acid produced when the nitrogen is supplied as nitrogen source 2 enriched with yeast extract at the rate of 0·15 %. Enrichment of the broth with higher concentrations of yeast extract has a slight influence on the amount of acid produced from glucose, mannose and salicin; but the increase in the amount of acid produced from these carbohydrates and from fructose is not appreciable even when enrichment of the broth with yeast extract is at the rate of 5 %—equivalent to 0·33 % total nitrogen. Actually, the enrichment with 5 % yeast extract appears to have an inhibiting effect on the fermentation of galactose. The fermentation of lactose is not affected when peptic caseinogen digest broth (0·5 % T.N.) is supplemented by yeast extract. The influence of the nitrogen source on the fermentation of galactose and lactose confirms our previous observation that "in some measure there is a protein-carbohydrate-streptococcus linking up" [Sadler *et al.*, 1932, 2].

Apart from the fermentation of mannitol, culture EMB_1 195 is strictly comparable with culture EMB_1 173 in its sugar-fermenting abilities, whether the nitrogen is supplied as peptic caseinogen digest broth (0·5 % T.N.) or as the same broth enriched with yeast extract as defined. For the fermentation of mannitol it would seem that culture EMB_1 195 requires nitrogen of the kind available in yeast extract—an observation again in harmony with our previous finding.

When peptic caseinogen digest broth (approx. 1 % T.N.) is employed as the nitrogen source, the total titratable acidity produced by cultures EMB_1 173 and 195 from fructose, glucose, mannose, galactose, lactose and salicin respectively is much greater than the amount produced when the same broth diluted to contain approximately 0·5 % total nitrogen is used, and previous results, where comparison is possible, are confirmed.

Both cultures produce a much higher total titratable acidity from fructose, glucose, mannose, lactose and salicin when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen than is produced when the diluted broth enriched with yeast extract as defined is supplied.

Table I. *Titratable acidity in g. lactic acid per litre.*

Culture No.	Nitrogen source	Mannitol	Fructose	Glucose	Mannose	Galactose	Lactose	Salicin
				Coccus-forms.				
EMB$_1$ 173	2	2·5	5·0	4·7	4·5	3·6	4·5	3·4
		2·3	5·0	5·0	5·0	3·6	4·5	3·8
	2 *a*	2·7	5·4	5·2	5·0	3·6	4·7	4·3
	2 *b*	3·4	5·0	5·4	4·5	3·4	4·5	5·2
		3·4	5·6	5·6	5·4	4·3	4·7	5·2
	2 *c*	3·2	5·6	6·1	5·9	4·3	4·5	5·6
	2 *d*	3·6	6·5	6·1	6·3	2·0	4·7	5·2
	1	3·8	8·3	8·3	7·9	7·4	7·0	6·1
EMB$_1$ 195	2	0·0	4·7	5·0	4·5	3·4	4·5	4·1
		0·2	5·2	4·7	4·5	3·2	4·3	3·2
	2 *a*	2·5	4·7	5·0	4·7	3·2	4·1	3·8
	2 *b*	1·6	5·0	5·4	5·2	3·4	4·3	4·3
		2·0	5·2	5·2	5·0	3·2	4·5	4·3
	2 *c*	1·1	5·9	5·4	5·9	4·1	4·5	3·8
	2 *d*	3·2	6·8	5·9	6·5	2·3	5·0	5·4
	1	0·7	7·4	7·7	7·9	7·2	6·5	5·9
EMB$_2$ 168	2	0·2	4·5	5·0	5·9	5·4	5·6	0·7
	2 *b*	0·2	7·7	7·7	8·3	7·7	6·8	0·0
	2 *d*	0·5	9·2	7·7	9·7	6·3	8·6	2·0
	1	0·9	7·0	8·3	7·9	9·0	8·8	0·7
EMB$_2$ 173	2	0·2	3·2	4·5	5·2	4·3	5·0	0·0
		0·0	4·1	5·2	5·0	4·5	5·9	1·4
	2 *a*	0·2	6·1	7·0	7·7	5·4	7·0	0·5
	2 *b*	0·5	8·8	8·3	8·6	7·2	7·9	5·9
		0·5	7·9	6·8	7·9	7·2	7·2	3·6
	2 *c*	0·2	7·2	7·2	8·3	7·2	7·4	4·3
	2 *d*	0·5	9·5	8·6	9·9	7·4	8·8	7·0
	1	0·2	7·4	8·1	8·6	8·3	9·9	3·2
				Rod-forms.				
EMB$_1$ 58	2	2·0	6·5	5·6	5·6	5·2	4·5	7·4
		2·5	7·2	5·4	7·2	5·4	5·9	6·1
	2 *a*	2·7	8·6	7·4	9·7	7·7	8·8	8·6
	2 *b*	3·4	9·9	8·8	11·3	9·2	9·7	9·7
		3·8	9·7	9·7	11·9	10·6	9·5	9·5
	2 *c*	3·8	10·6	9·9	12·4	—	10·1	9·5
	2 *d*	6·1	10·8	11·7	14·0	8·6	11·7	11·3
	1	2·5	11·9	10·4	11·9	10·4	8·8	11·0
EMB$_2$ 189	2	2·5	7·0	7·9	9·2	7·4	9·0	8·8
		2·3	8·8	6·8	9·7	6·5	7·7	8·6
	2 *a*	2·7	9·9	9·2	10·4	6·5	9·5	8·3
	2 *b*	3·4	11·0	10·4	11·7	9·7	10·1	9·7
		3·6	11·5	10·8	11·7	10·8	10·4	9·9
	2 *c*	5·6	11·9	11·3	12·8	10·8	10·8	9·5
	2 *d*	6·8	12·8	12·6	14·2	9·0	12·8	12·2
	1	2·9	15·3	15·1	14·6	12·4	14·2	11·5
EMB 33	2	2·3	7·0	7·2	9·5	7·0	8·1	9·0
		2·5	7·9	6·8	8·8	6·1	7·2	8·3
	2 *a*	2·3	8·6	6·8	9·5	6·5	8·3	—
	2 *b*	3·4	10·6	9·5	11·7	10·1	8·8	9·0
		3·6	9·9	9·5	10·7	8·6	9·5	9·0
	2 *c*	3·8	11·3	9·7	11·7	9·0	10·4	9·5
	2 *d*	5·6	11·9	11·9	13·5	8·8	11·9	11·9
	1	3·2	14·4	15·1	14·9	12·6	14·2	13·1

Nitrogen sources:

2 Peptic caseinogen digest broth containing approx. 0·5 % total nitrogen [Eagles and Sadler, 1932].

2 *a* Nitrogen source 2 enriched with Bacto yeast extract at the rate of 0·15 %—equivalent to an enrichment of 0·01 % total nitrogen [Eagles and Sadler, unpublished results].

2 *b* Nitrogen source 2 enriched with Bacto yeast extract at the rate of 1 %—0·066 % T.N.

2 *c* Nitrogen source 2 enriched with Bacto yeast extract at the rate of 2 %—0·132 % T.N.

2 *d* Nitrogen source 2 enriched with Bacto yeast extract at the rate of 5 %—0·33 % T.N.

1 Peptic caseinogen digest broth containing approx. 1 % total nitrogen [Eagles and Sadler, 1932].

When employing nitrogen source 2 enriched with 0·15 % yeast extract for determining the sugar-fermenting abilities of culture EMB_2 173, the total titratable acidity produced from fructose, glucose and mannose respectively is more than 50 % higher than is produced when the same nitrogen source without yeast extract enrichment is used; and, although less marked, there is an increase in the total titratable acidity produced from galactose and lactose when the enriched broth is employed. When the basic broth is enriched with higher concentrations of yeast extract the increase in the total titratable acidity produced by the culture from fructose, glucose, mannose and lactose respectively is marked, particularly when enrichment is at the rate of 5 %; even so, the critical differences in the total titratable acidity produced from fructose, glucose, mannose and lactose are to be seen when comparing the influence of nitrogen source 2 and nitrogen source 2 enriched with a trace only (0·15 %) of yeast extract. Throughout, the culture is unable to ferment mannitol. On the other hand, salicin, which is not fermented by the culture when the nitrogen is supplied as peptic caseinogen digest broth (0·5 % T.N.) or as the same broth enriched with 0·15 % yeast extract, is fermented to a marked degree when the broth is enriched with yeast extract at the rate of 5 %, an indication of the protein-carbohydrate-streptococcus linking up shown before [Sadler *et al.*, 1932, 2].

Culture EMB_2 168 ferments salicin feebly to acid, only when the nitrogen source is peptic caseinogen digest broth (0·5 % T.N.) enriched with yeast extract at the rate of 5 %, and, in this respect, differs from culture EMB_2 173. Otherwise, as far as comparison is possible, nitrogen sources 2*a* and 2*b* not having been used for culture EMB_2 168, cultures EMB_2 168 and 173 are closely similar in their ability to ferment the carbohydrates employed in the study.

The total titratable acidity produced by cultures EMB_2 173 and 168 from fructose, glucose, mannose, galactose and lactose is about 100 % higher when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen rather than the same broth diluted to contain approximately 0·5 % T.N.; and our previous results when glucose, mannose and lactose only were submitted to fermentation, are confirmed. It is also seen that, responding as they do to enrichment of the diluted broth with small amounts of yeast extract, these cultures, nevertheless, produce from fructose, glucose, galactose and lactose almost as much acidity when the nitrogen source is peptic caseinogen digest broth (approx. 1 % T.N.) as is produced when the same broth diluted (approx. 0·5 % T.N.) is enriched with yeast extract at the rate of 5 %. For the fermentation of salicin, on the other hand, peptic caseinogen digest broth, total nitrogen content 1 or 0·5 %, is not a suitable nitrogen source; but a greater or less amount of acid is produced when enrichment of the broth with yeast extract is provided—an indication of protein-carbohydrate-streptococcus linking up [Sadler *et al.*, 1932, 2].

When the peptic caseinogen digest broth containing approximately 0·5 % total nitrogen is enriched with yeast extract at the rate of 0·15 %, the total titratable acidity produced by culture EMB_1 58 from glucose, mannose, galactose and lactose is much higher than is the case when the same broth without enrichment is employed as the nitrogen source—the increase in total acidity being particularly evident in the fermentation of mannose and lactose. The influence of the enrichment on the increase in the total acidity produced from glucose, mannose and lactose is more marked as higher concentrations of yeast extract are made available; and when the broth enriched with yeast extract at the rate of 5 % is used as the nitrogen source there is an increase in the total titratable acidity of over 200 %. In the fermentation of mannitol, the total titratable acidity produced is comparatively low; but, comparing the influence of the basic broth and the same broth enriched with yeast extract at the rate of 5 %, there is seen to be an increase of 300 % in the amount of acid produced when the latter nitrogen source is employed. The influence of yeast extract enrichment on the fermentation of galactose is to be seen in the marked successive increases in the amount of acid produced when the nitrogen sources are *2a* and *2b* respectively; but enrichment with yeast extract at the rate of 5 % provides a less suitable nitrogen source than enrichment at the rate of 1 %. In the fermentation of salicin, enrichment of the basic nitrogen source with yeast extract provides for an increase in the total titratable acidity produced; yet the increase is not more than approximately 20 % until the concentration of the enrichment is 5 %.

When peptic caseinogen digest broth containing approximately 1 % total nitrogen is used as the nitrogen source, the total titratable acidity produced from fructose, glucose, mannose, galactose, lactose and salicin is from 50 % to 100 % higher than the amounts produced when the nitrogen is supplied as the same broth diluted to contain approximately 0·5 % total nitrogen; and our previous results [Sadler *et al.*, 1932, 1] in the study of lactic acid-producing coccus types are confirmed.

For the fermentation of mannitol, the peptic caseinogen digest broth containing approximately 1 % total nitrogen is no more suitable as a nitrogen source than is the same broth diluted to contain approximately 0·5 %; and, as a nitrogen source, it cannot be compared with the diluted broth enriched with yeast extract at the rate of 5 % (0·33 % T.N.), the suspicion thereby arising that, as has been shown already for the lactic acid streptococci [Sadler *et al.*, 1932, 2], the lactic acid-producing rods may require a certain moiety of a particular nitrogen fraction for the fermentation of a particular carbohydrate.

When the nitrogen is supplied as peptic caseinogen digest broth containing approximately 0·5 % total nitrogen, cultures EMB_2 189 and EMB 33 produce a higher total titratable acidity from fructose, glucose, mannose, lactose and salicin than is produced by culture EMB_1 58. There is no further increase in the amount of acid produced by culture EMB 33 when the nitrogen source broth is enriched with yeast extract at the rate of 0·15 %, and the influence of the same enrichment on the sugar-fermenting abilities of culture EMB_2 189 is only slight. The increase in the total titratable acidity produced by both cultures from fructose, glucose, mannose and lactose is appreciable as the basic broth is enriched with yeast extract at the rate of 1, 2 and 5 % respectively; but the influence of enrichment is not marked until the yeast extract concentration reaches 5 %, an effect which is also reflected definitely in the fermentation of salicin. Cultures EMB_2 189 and EMB 33 display the same idiosyncrasy to the fermentation of galactose, employing nitrogen source 2 enriched with yeast extract at the rates of 1 and 2 % as is shown by culture EMB_1 58; and, throughout, mannitol is attacked in like manner by the three cultures.

Whether the total nitrogen content of the peptic caseinogen digest broth supplied as the nitrogen source be 0·5 or 1 %, it would appear that cultures EMB_2 189 and EMB 33 are more active and vigorous than cultures EMB_1 58, if activity and vigour may be interpreted in terms of the total titratable acidity produced by each culture from fructose, glucose, mannose, galactose, lactose and salicin; or, it may be that, in the light of our previous results [Sadler *et al.*, 1932, 1], peptic caseinogen digest broth of either 0·5 or 1 % total nitrogen content is a more suitable nitrogen source for cultures EMB_2 189 and EMB 33 than for culture EMB_1 58, interpreting suitability in terms of the total titratable acidity produced by each culture from fructose, glucose, mannose, galactose, lactose and salicin respectively.

An increase of from 50 to 100 % in the total titratable acidity produced by cultures EMB_2 189 and EMB 33 from fructose, glucose, mannose, galactose and lactose is to be seen, and the increase in the amount of acid produced from salicin is marked when the nitrogen source is peptic caseinogen digest broth containing approximately 1 % total nitrogen rather than the same broth diluted to contain approximately 0·5 % total nitrogen, again confirming previous results.

SUMMARY.

Considering the results as a whole it is seen that for two of the four coccus strains—cultures EMB_1 173 and 195—enrichment of peptic caseinogen digest broth (approx. 0·5 % T.N. content) with yeast extract at the concentrations defined has only a slight influence on the total titratable acidity produced from fructose, glucose, mannose and salicin even when enrichment is at the rate of 5 %, equivalent in terms of total nitrogen to 0·33 %; and the influence of the nitrogen source on the fermentation of galactose and lactose confirms our previous observation [Sadler *et al.*, 1932, 2].

For two of the coccus strains—cultures EMB_2 173 and 168—the total titratable acidity produced from fructose, glucose, mannose, galactose and lactose is at least 50 % higher when peptic caseinogen digest broth (approx. 0·5 % T.N.) enriched with yeast extract at the rate of 1 % rather than the same broth without enrichment is employed as the nitrogen source, the increase in the amount of acid produced being more marked as the broth is enriched with higher concentrations of yeast extract.

Of the three rod-forms, culture EMB_1 58 produces a much higher total titratable acidity from glucose, mannose, galactose and lactose when the nitrogen source is peptic caseinogen digest broth (0·5 % T.N.) enriched with yeast extract at the rate of 0·15 % instead of the same broth without enrichment. Enrichment with yeast extract at the rate of 0·15 % fails to influence the sugar-fermenting abilities of culture EMB 33 and influences only slightly the sugar-fermenting abilities of culture EMB_2 189: yet both cultures produce a higher total titratable acidity from fructose, glucose, mannose and salicin when the nitrogen is supplied as peptic caseinogen digest broth (0·5 % T.N.) without enrichment than is produced by culture EMB_1 58 when the same nitrogen source is employed. Increasing the concentration of yeast extract enrichment reflects itself in the amount of acid produced from certain carbohydrates by each of the three cultures; and for the fermentation of such carbohydrates the increase in the total titratable acidity is marked when the yeast extract concentration reaches 5 %.

In the fermentation of mannitol the total titratable acidity produced by cultures EMB_1 58, EMB_2 189 and EMB 33 is comparatively low throughout; but comparing the influence of the basic broth and the same broth enriched with yeast extract at the rate of 5 %, there is seen to be an increase of 300 % in the amount of acid produced when the latter nitrogen source is employed. The idiosyncrasy to the fermentation of galactose by each of the three cultures is well defined.

For the fermentation of mannitol, the peptic caseinogen digest broth containing approximately 1 % total nitrogen is no more suitable as a nitrogen source than is the same broth diluted to contain approximately 0·5 % total nitrogen, and, as a nitrogen source, it cannot be compared with the diluted broth enriched with yeast extract at the rate of 5 % (0·33 % T.N.), the suspicion thereby arising that, as has been shown already for the lactic acid streptococci, the lactic acid-producing rods may, likewise, require a certain moiety of a particular nitrogen fraction for the fermentation of a particular carbohydrate.

For the fermentation of fructose, glucose, mannose, galactose and lactose by the 7 cultures studied—coccus forms and rod forms—the total titratable acidity is increased by 50 to 100 % when employing as the nitrogen source a peptic caseinogen digest broth containing approximately 1 % total nitrogen instead of the same broth diluted to contain 0·5 % (approx.) total nitrogen, previous results being thus confirmed.

In supplying the nitrogen for the fermentation of certain carbohydrates by each of the organisms under study, a stimulating influence equal to that caused by an enrichment of the diluted peptic caseinogen digest broth (approximately 0·5 % T.N.) with yeast extract at the rate of 5 % (0·33 % T.N.) may be assured by employing peptic caseinogen digest broth alone, providing the total nitrogen content is approximately 1 %.

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CII. ON THE ASSAY AND THE ABSORPTION OF TESTICULAR HORMONE DISSOLVED IN OIL.

By VLADIMIR KORENCHEVSKY, MARJORIE DENNISON
AND ALICE KOHN-SPEYER.

From the Lister Institute, London.

(*Received May 1st, 1933.*)

In a previous paper [Korenchevsky, Dennison and Kohn-Speyer, 1933] it was stated that the assay of testicular hormone on rats had given accurate results in our experiments, in spite of the fact that, on autopsy, some unabsorbed oil was found in the subcutaneous tissue in the places where the injections had been more recently made; an appreciable amount being found at the site of the last 2 days' injections.

Because of the accurate results obtained in the assay it was suggested that, with the doses used, either the oil is absorbed from the subcutaneous tissue at a constant rate, or the testicular hormone is more readily absorbed from the oil than is the oil itself. If these assumptions were incorrect, very variable results would be expected.

This is of especial importance in the assay of the rat unit, as proposed by us, since the injections have to be made during a period of only 7 days, *i.e.* during a short period, in which, if the rate of absorption varied, much more variable results would be expected than in experiments of long duration. Moreover, if the testicular hormone during the last 2 or 3 days of the experiment is not absorbed from the subcutaneous tissue, it would be advisable for the assay to inject the rats for the first 4 days only, to wait for the following 3 days for a complete absorption of the oily solution from the subcutaneous tissue, and only then to kill the rats after 7 days of experiment, *i.e.* 3 days after the last injection.

For the elucidation of the question, whether the hormone injected in the second half of the assay period is absorbed, and whether perhaps only 4 days' injections are sufficient to give approximately the same result as 7 days', the present experiments were performed. At the same time, we have used the results of these experiments and of some others in order to compare again the standardisation of the hormone preparation by the capon comb-growth method and by our own method.

Technique.

The experiments were performed on 15 rats belonging to 3 litters (Table I). Litters 1 and 2 were each divided into 3 groups of rats: group I, control rats; group II, rats injected daily with 2·5 c.u. of testicular extract on the first 4 days of the experiment and then left without injections during the last 3 days; group III, rats injected with the same daily dose of extract on each of the 7 days of the experiment. The rats of litter 3 were divided into 2 groups only: control rats and those injected for 7 days. All the rats were killed on the 8th day. In other respects the technique used was the same as that suggested for the assay of the rat unit [Korenchevsky *et al.*, 1932]. The diet used was the same as that described in our previous paper [Korenchevsky *et al.*, 1933]. The testicular

Table I. *Age and average weight of the castrated rats in each group of each litter.*

		Age in days			No. of days after castration at beginning of injections	Final weight		
No. of litter	No. of rats in litter	At castration	When injections started	Final		Control rats g.	4 days injected g.	7 days injected g.
1	6	24	64	71	40	246	252	287
2	5	25	67	74	42	217	262	244
3	4	28	46	53	18	183	—	174

hormone was prepared by Messrs Schering, Ltd.[1] by extraction from urine and was dissolved in sesame oil. It was of a different batch from that used in our previous experiments [Korenchevsky *et al.*, 1932] and the strengths in rat units of these two batches will be compared below (Table III). This hormone preparation had been standardised by Messrs Schering, Ltd., by the method of comb-growth in capons and was stated to contain 2·5 C.U. in 0·1 cc. as in previous batches. Unfortunately, the amount of preparation available was only sufficient for experiments on three litters.

The influence on prostate with seminal vesicles and on penis.

We had at our disposal only two litters, Nos. 1 and 2 (Table I), which were of similar age, castrated at about the same period before sexual maturity and kept for about 41 days after castration, *i.e.* which fulfilled all the requirements for assaying the rat unit as previously defined [Korenchevsky *et al.*, 1932, p. 2106]. The third litter was younger and the rats weighed less, and, although they were castrated at a similar age to that of litters 1 and 2, only 18 days elapsed between the date of castration and the date on which injections were started, instead of from 30 to 50 days as is required for the assay. We, therefore, took the average of the results of the first 2 litters only, using the third litter merely to show that 7 days' injections give altogether different results from those obtained when 4 days' injections are made. However, in spite of the difference in age and in the length of time after castration, the increase in the weight of the prostate with seminal vesicles was very similar to that obtained in the rats of litters 1 and 2 (see Table II), and therefore, even if we use all 3 litters for the average, the percentage increase of these organs calculated per 200 g. of body weight is nearly the same.

Since the rats differed in weight, only the results calculated per 200 g. of body weight are discussed below.

From Table II it is quite clear that if the injections were given for 7 days, the increase in the weight of prostate with seminal vesicles was about 3 times as great, and the increase in the weight of penis was about 4 times as great, as that of the respective organs in the rats injected during the first 4 days only. This indicates that the testicular hormone, injected in doses of 0·1 cc. of oily solution, was absorbed during the whole period of injection, possibly at a definite rate per day. In our previous experiments the effect of the injections appeared to be proportionate not only to the size of the dose but probably also to the number of days of injection [Korenchevsky *et al.*, 1933]. Therefore, taking into consideration the average percentage increase per day of sexual organs after 7 days' injections, the effect of 4 days' injections was much smaller than would

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[1] We wish to express our thanks to Messrs Schering, Ltd., for kindly supplying the preparation.

Table II. *Influence on prostate with seminal vesicles, penis, adrenals and thymus.*

The average weights (mg.), actual and calculated per 200 g. of body weight, of the organs of the rats in each group of each litter and their percentage change after the injection of testicular hormone.

	Actual weights					Weights per 200 g. of body weight				
	Con- trol	Rats injected		% change		Con- trol	Rats injected		% change	
No. of litter	rats	4 days	7 days	4 days	7 days	rats	4 days	7 days	4 days	7 days
Prostate with seminal vesicles										
1	66	88	162	+33·3	+145·4	54	70	114	+29·6	+111·1
2	53	87	122	+64·2	+130·1	49	66	100	+34·7	+104·1
Average	**60**	**88**	**142**	**+48·8**	**+137·8**	**52**	**68**	**107**	**+32·2**	**+107·6**
3	64	—	115	—	+ 79·7	70	—	133	—	+ 90·0
Penis										
1	84	97	141	+15·5	+67·8	68	77	99	+13·2	+45·6
2	72	100	120	+38·9	+66·7	66	74	98	+12·1	+48·5
Average	**78**	**99**	**131**	**+27·2**	**+67·3**	**67**	**76**	**99**	**+12·7**	**+47·1**
3	71	—	111	—	+56·4	77	—	128	—	+66·2
Adrenals										
1	100·8	79·6	89·6	−21·0	−11·1	82·0	63·3	62·4	−22·8	−23·9
2	85·4	84·2	72·8	− 1·5	−14·7	78·7	64·4	59·1	−18·2	−24·9
Average	**93·1**	**81·9**	**81·2**	**−11·3**	**−12·9**	**80·4**	**63·9**	**60·8**	**−20·5**	**−24·4**
3	70·5	—	65·0	—	− 7·8	77·3	–	75·0	—	− 3·0
Thymus										
1	604	478	582	−20·9	− 3·6	491	380	405	−22·6	−17·5
2	637	497	597	−21·9	− 6·3	587	382	488	−34·9	−16·9
Average	**621**	**488**	**590**	**−21·4**	**− 5·0**	**539**	**381**	**447**	**−28·8**	**−17·2**
3	721	—	548	—	−24·0	786	—	631	—	−19·8

be expected. Such a slight effect is probably explained by the fact that after the cessation of injections the weight of the sexual organs, which was increased by 4 days' injections, started to revert towards the weight of the pre-injection period, owing to the lack of testicular hormone in the organism. Thus, cessation of the injections in castrated animals produced an effect on the sexual organs which is similar to that obtained by removal of the testes in normal animals, and possibly equally rapid.

The influence on adrenals and on thymus.

It was found before that after 7 days' injections, besides the sexual organs, only the thymus and occasionally to a slight extent the adrenals, thyroid and hypophysis of castrated rats showed changes towards the normal condition. In the present experiments, after injection there were noticeable changes in the involution of the thymus and in the decrease in size of the hypertrophied adrenals. Both these changes were, however, slight and irregular and independent of the number of days of injection. In litter 3 changes in the adrenals were negligible.

In all other organs and in the appetite and rate of growth of the rats, no regular changes were observed.

Comparison of assays of the rat unit and capon unit.

For this purpose we have summarised in Table III data obtained in the assay of two batches of testicular hormone. The results obtained in the present experiment with batch B on litters 1 and 2 were compared with those obtained

Table III. *Number of hormone units in two batches of testicular extract as assayed by the authors' method on rats and as assayed by the comb-growth method on capons.*

		Average age in days		Average percentage increase per 200 g. of body weight				No. of units	
				Prostate with seminal vesicles		Penis			
No. of batch of extract	Average final weight of rats g.	At castration	Final	Total	Per 1 c.u.	Total	Per 1 c.u.	By capon method	By rat method
Batch A	193	28	67	88·4	35·4	38·7	15·5	2·5	2·21 $\left(\frac{88{\cdot}4}{40}\right)$
Batch B	251	25	73	107·6	43·0	47·1	18·8	2·5	2·69 $\left(\frac{107{\cdot}6}{40}\right)$

in a previous paper [Korenchevsky *et al.*, 1932, Table I, p. 2097 and Table V, p. 2101] on litters 4 and 6 injected with the same daily dose of testicular hormone of batch A.

Each batch was assayed on two litters only, but in spite of the small number of litters used in the assay, the results obtained by both methods agreed satisfactorily. Thus, the strength of both batches, using the capon method, was marked by Schering, Ltd., to be 2·5 c.u. in 0·1 cc. of the preparation, whilst we have found the strength of batch A to be 2·21 r.u. and of batch B to be 2·69 r.u. in 0·1 cc. This is only 11·6 % less and 7·6 % more respectively than the number (2·5) of capon units indicated. Using our method, in some batches, however [see for example, Korenchevsky *et al.*, 1933, p. 563, Table V] the strength of the extract was found to be nearly twice as great as that given by the capon method, whilst in others again it was quite close to the figure indicated. Since, in the latter experiments the period of injection and the time elapsed since castration were longer, they will be discussed elsewhere.

Summary.

1. Experiments were performed on 15 castrated male rats belonging to 3 litters, and the results were compared with those previously published.
2. The effect of 7 days' injection of testicular hormone preparation was about 3 times as great on prostate with seminal vesicles, and about 4 times as great on penis, as that on the respective organs in the litter-mates, injected during the first 4 days only, but killed simultaneously with the former group of rats on the 8th day.
3. In these rats the increase in weight of the sexual organs obtained after 4 days' injections was smaller than would be expected considering the total dose injected and the number of days of injection.
4. It is, therefore, most probable that the weight of the sexual organs, which was increased by 4 days' injections, after cessation of the injections started to return towards the weight of the pre-injection period, owing to the lack of continued supply of hormone in the organism.
5. The above data, together with the accuracy and similarity of the results obtained in the present as well as in previous experiments, indicate that in rats the hormone was absorbed from oily solution (injected in doses of 0·1 cc.) during the whole period of injection at some definite constant rate of absorption.

6. A comparison of the content of rat units and of capon units in two different batches of testicular hormone gave similar figures, showing that the rat unit was nearly equal to the capon unit in these extracts.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due.

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CIII. THE INFLUENCE OF TESTICULAR HORMONE ON CRYPTORCHID RATS.

By VLADIMIR KORENCHEVSKY, MARJORIE DENNISON
AND ALICE KOHN-SPEYER.

From the Lister Institute, London.

(*Received May 1st, 1933.*)

In previous papers [see references Korenchevsky, Dennison and Kohn-Speyer, 1933] the influence of a testicular hormone preparation (isolated from human urine and highly purified) was studied on castrated and young normal rats. In the present investigation the same preparation was injected into cryptorchid rats, in whose testes all seminiferous tissue, except the Sertoli cells, is degenerate, while the Leydig cells appear to remain normal. Thus, the aim of the present experiments was to ascertain the effect of testicular hormone, when injected for a short period, on the weight of organs of animals, whose testes do not contain any normal seminiferous tissue except Sertoli's cells.

Technique.

13 rats belonging to 3 litters were made cryptorchid in the usual way [Korenchevsky, 1930]. Each litter was divided into a group of control rats (total 7 rats) and a group of injected rats (total 6 rats). The same technique was used, and the same preparation of Messrs Schering, Ltd.[1], was injected as in previous experiments [Korenchevsky, Dennison and Kohn-Speyer, 1933], the batch of extract being the same as that designated "II" and employed for litters 9 to 12 of normal rats and litters 1 to 4 of castrated rats. A large daily dose of 5 C.U. (as standardised by Messrs Schering, Ltd.) was injected for 10 days, in half doses twice a day. Such a dose produces quite definite changes both in castrated and normal animals. The final age of the rats, the age at which they were operated, and the final weights of the rats are given in Table I.

Table I. *Age and average weight of the cryptorchid rats in each group of each litter.*

No. of litter	No. of rats in litter	Age in days			No. of days after operation at beginning of injections	Final weight	
		At operation	When injections started	Final		Control rats g.	Injected rats g.
1	4	33	122	132	89	321	336
2	4	47	110	120	63	351	381
3	5	27	90	100	63	343	351

Influence on testes.

Without histological investigation it is difficult to estimate quite definitely the significance of changes in weight of cryptorchid testes, since these are usually more or less oedematous, and the weight of the testes varies according to the degree of oedema found. We always noted the condition of the testes. If the weights of those which were unusually oedematous were excluded from our calculations, we found that the testes of most of the injected rats were slightly smaller than those of the controls. The average percentage change in weight is shown in Table II.

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[1] We wish to express our thanks to Messrs Schering, Ltd., for kindly supplying the preparation.

Table II. *Influence on testes, prostate with seminal vesicles, penis and thymus.*

The average weights, actual and calculated per 200 g. of body weight, of the organs of the rats in each group of each litter and their percentage change after the injection of testicular hormone as compared with those of the control rats.

	Actual weights			Weights per 200 g. of body weight		
	Control rats	Injected rats		Control rats	Injected rats	
No. of litter	g.	g.	% change	g.	g.	% change
Testes						
1	0·734	0·800	+ 9·0	0·460	0·488	+ 6·1
2	1·334	0·755	−43·4	0·762	0·397	−47·9
3	0·978	0·612	−37·4	0·565	0·346	−38·8
Average	**1·015**	**0·722**	**−23·9**	**0·596**	**0·410**	**−26·9**
Prostate with seminal vesicles						
1	1·651	1·305	−20·9	1·028	0·783	−23·8
2	1·622	1·384	−14·7	0·924	0·728	−21·2
3	1·309	1·076	−17·8	0·763	0·614	−19·5
Average	**1·527**	**1·255**	**−17·8**	**0·905**	**0·708**	**−21·5**
Penis						
1	0·334	0·365	+ 8·7	0·209	0·219	+ 4·8
2	0·368	0·354	− 3·8	0·210	0·186	−11·4
3	0·319	0·342	+ 7·2	0·186	0·195	+ 4·8
Average	**0·340**	**0·354**	**+ 4·0**	**0·202**	**0·200**	**− 0·6**
Thymus						
1	0·368	0·248	−32·6	0·229	0·145	−36·7
2	0·355	0·303	−14·6	0·203	0·160	−21·2
3	0·466	0·408	−12·4	0·269	0·231	−14·1
Average	**0·396**	**0·320**	**−19·9**	**0·234**	**0·179**	**−24·0**

Influence on prostate with seminal vesicles.

In all the injected rats, without exception, these glands were smaller than those of the control uninjected rats (Table II). Therefore, in spite of the smallness of the decrease in the weight, it may be concluded that the injections depressed the development of the prostate and seminal vesicles.

Influence on other organs, fat deposition and appetite.

Whilst the involution of the thymus (Table II) was slightly accelerated, no significant or constant changes were observed in the penis (Table II), adrenals, thyroid, hypophysis, liver, kidneys, spleen, heart or fat deposition; nor was any considerable effect noted in the rate of growth and appetite of the rats. The latter data, therefore, were not included in the tables.

DISCUSSION.

On the basis of the definite results obtained there is no doubt that a testicular extract, isolated from urine, which had a stimulating action on the sexual organs of castrated rats, had no stimulating effect on the sexual organs of cryptorchids. On the contrary, a depressing effect, although slight, was noted on the prostate with seminal vesicles, and probably also on the testes.

On the average, the weight of the prostate with seminal vesicles of cryptorchid rats is usually slightly smaller than that of normal rats of a similar age [Korenchevsky, 1930]. For instance, in normal rats, whose age was similar to that of the rats of the present experiments, the weight of the prostate with seminal vesicles varied between 1·25 g. and 1·94 g. per 200 g. of body weight [Korenchevsky, 1930, Table IV, p. 629 and Table IX, p. 634]. In the present experiments these organs in the control uninjected rats weighed from 0·763 to 1·028 g. and in the injected rats from 0·614 to 0·783 g., *i.e.* considerably less than in the normal rats. It was, therefore, contrary to expectation to observe no stimulating effect of the testicular hormone in cryptorchid rats, as such a stimulating effect was always noted in castrated rats. The effect obtained was similar to that found with some doses of the hormone on the testes and on the prostate with seminal vesicles of young normal rats [Korenchevsky *et al.*, 1933, pp. 569, 570]. These results indicate the presence in the testicular extract isolated from urine of some property or substance inhibiting the development of sexual organs in animals. Such an inhibitory effect became evident when certain doses of extract were injected into animals possessing testes. The experiments performed do not indicate whether this inhibiting effect was due to the direct influence of the extract or to indirect influence of the extract through some endocrine organs.

SUMMARY.

1. In the experiments performed on 13 cryptorchid rats, a testicular hormone preparation, isolated from urine and injected subcutaneously, did not stimulate their sexual organs, but as judged by changes in weight of these organs had a slight inhibitory effect on the development of the prostate with seminal vesicles and probably also of the testes.

2. Under the influence of the injections the involution of the thymus was slightly accelerated.

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CIV. STUDIES IN TISSUE METABOLISM.

I. VITAMIN B_1 AND THE COENZYME OF LACTIC DEHYDROGENASE.

By ERIC BOYLAND.

From the Research Institute of the Cancer Hospital (Free), London.

(*Received May 1st, 1933.*)

The suggestion that the antiberiberi factor was concerned with carbohydrate metabolism arose early in vitamin work [Braddon and Cooper, 1914; Funk and Schönborn, 1914] and has received much attention since. It is only within the last few years, however, that work carried out by Peters and his collaborators has defined the rôle of alkali-labile vitamin B_1. Kinnersley and Peters [1929; 1930] showed that the brains of polyneuritic pigeons had a relatively high lactic acid content, and Gavrilescu and Peters [1931, 1] found that the brain tissue of avitaminous polyneuritic pigeons had a sub-normal oxygen uptake *in vitro*. Soon afterwards [1931, 2] they showed that the addition of vitamin B_1 concentrates increased the *in vitro* respiration of this polyneuritic brain tissue. Meiklejohn, Passmore and Peters [1932, 1] have shown that the oxidation affected in this way is that of lactic acid, indophenol oxidase and succinic dehydrogenase being unaffected. They have also [1932, 2] been able to show that when polyneuritis of avitaminosis was cured by vitamin B_1 concentrates, the ability of the brain to increase its oxygen uptake in the presence of lactate returned. The action of vitamin B_1 concentrates containing a heat-stable substance, which stimulates oxidation of lactate, is like the action of a coenzyme added to an enzyme deficient in its coenzyme.

Recently Banga and Szent-Györgyi [1932] have described a coenzyme of lactic acid dehydrogenase. When washed heart muscle alone is incubated with lactate and methylene blue, the latter is not reduced. Methylene blue is not reduced by coenzyme alone or by lactate alone in the presence of washed heart muscle. If, however, heart muscle extract or the purified coenzyme obtained from such an extract is added to the washed muscle, it is then able to oxidise lactate in the presence of methylene blue. Anaerobically methylene blue is decolorised, aerobically oxygen is taken up [Banga, Szent-Györgyi and Vargha, 1932].

In the experiments of Gavrilescu *et al.* [1932] it was found that addition of lactate doubled the respiration of normal brain, but that the respiration of avitaminous brain was only increased by a quarter by addition of lactate. Addition of vitamin B_1 concentrates to normal brain in presence of lactate increased respiration by 4 % on the average, but with avitaminous brain the respiration was increased 20–32 %. The deficient respiration of avitaminous brain is peculiar to lactate metabolism. In these experiments the avitaminous brain appears as an enzyme deficient in coenzyme and the vitamin concentrates act as coenzyme.

Szent-Györgyi's coenzyme and vitamin B_1 are similar to some extent in chemical properties. The coenzyme is described as an adenine-like derivative containing sulphur; it is not adenyl pyrophosphate. Vitamin B_1 is stated to be a nitrogenous base containing sulphur.

An obvious difference between the lactic oxidase coenzyme described by Szent-Györgyi and the purest vitamin B_1 preparation is that the former is described as being yellow-coloured while vitamin B_1 is colourless. Preparations of coenzyme, however, have been made, both by the original technique and by modified methods, which are colourless and yet active. Banga, Szent-Györgyi and Vargha [1932] suggested that the activity ran parallel with colour in different preparations. The fact that the active coenzyme could be obtained colourless suggested that it might be identical with the antineuritic factor of yeast.

In order to test this hypothesis, crude and purified preparations of vitamin B_1 were tested for coenzyme activity in the presence of washed heart muscle, lactate and methylene blue by means of the Thunberg technique. Crude vitamin B_1 concentrates activated the lactic dehydrogenase of washed heart muscle, but purified preparations did not. A purified preparation of heart muscle coenzyme was then tested for vitamin B_1 activity by means of the rat curative test and was found to have no vitamin activity.

EXPERIMENTAL.

Lactic dehydrogenase coenzyme.

Two bullocks' hearts were obtained from the slaughter-house and stored in ice until used. The hearts were treated within 1½ hours after death. The heart muscle separated from fat and connective tissue was minced and the minced tissue (3 kg.) stirred into 3 litres boiling 1 % trichloroacetic acid. After 10 minutes' stirring the material was pressed through muslin and the extract filtered. The filtrate (which had coenzyme activity) was treated with excess of mercuric nitrate dissolved in nitric acid. The precipitate so formed was filtered off, washed with acetone and then extracted with HCl containing $HgCl_2$. The acid extract and washings (50 cc.) were then poured into 500 cc. of dried acetone, which precipitated the coenzyme. It was centrifuged and collected, dissolved in water and treated with H_2S to remove mercury. The mercury sulphide formed a colloidal suspension which was precipitated by addition of one-third volume of alcohol. The colourless filtrate (60 cc.) was then aerated to remove H_2S, and the coenzyme in the colourless solution was precipitated with dried acetone (500 cc.), removed by centrifuging and dried in a vacuum desiccator.

Vitamin B_1 preparations.

The first crude vitamin B_1 concentrate was prepared in the Lister Institute (preparation A in Table II) from acetic acid extracts of brewer's yeast. The extract was treated with lead acetate, baryta, mercuric sulphate and then H_2S. The vitamin was adsorbed on norite at p_H 7·0, eluted with $N/10$ HCl and the extract neutralised. The second crude preparation (B) was made from baker's yeast in the Biochemical Laboratory, Oxford, as described by Kinnersley, O'Brien, Peters and Reader [1933]. Both preparations A and B contained 3 pigeon doses per cc. These crude concentrates were free from vitamin B_2. The purified vitamin preparations were made in the Biochemical Laboratory, Oxford. The preparation purified by phosphotungstate contained 3 pigeon doses per cc. and the crystalline preparation 2·5 doses per cc. (in 5γ solids). The crystals were prepared by the method described by Kinnersley, O'Brien and Peters [1933].

Comparison of vitamin B_1 and heart muscle extract as coenzyme.

20 g. of well-minced bullock's heart muscle were ground with 250 cc. ice-cold water and pressed through muslin. The residue was washed in this way 6 times, pressed and then frozen solid and suspended in 100 cc. $M/10$ phosphate buffer, p_H 7·4. While frozen it was minced 3 times to break up the tissue.

2 cc. lots of this suspension with 1 cc. 0·01 % methylene blue were placed in Thunberg tubes with additions shown in Table I, evacuated and incubated in a glass-sided thermostat at 37°. The reduction times are shown in Table I.

Table I. *The reduction of methylene blue by washed heart muscle in the presence of lactate and lactic dehydrogenase coenzyme or vitamin B_1 preparations.*

$M/20$ lactate cc.	Lactic dehydrogenase coenzyme solution 10 mg. per cc. cc.	Vitamin solution		Vitamin preparation	Reduction time in minutes
		cc.	Pigeon doses		
—	—	—	—	—	>200
1	—	—	—	—	180
—	0·1	—	—	—	200
—	0·2	—	—	—	180
—	0·4	—	—	—	150
1	0·1	—	—	—	47
1	0·2	—	—	—	27
1	0·4	—	—	—	12½
—	—	0·5	1·5	Crude A	70
—	—	1·0	3·0	,,	60
1	—	0·1	0·3	,,	47
1	—	0·5	1·5	,,	40
1	—	1·0	3·0	,,	27
—	—	0·1	0·3	Crude B	115
—	—	0·2	0·6	,,	50
—	—	0·4	1·2	,,	36
1	—	0·1	0·3	,,	42
1	—	0·2	0·6	,,	33
1	—	0·4	1·2	,,	25
—	—	0·2	0·6	Purified by phosphotungstate	>200
—	—	0·4	1·2	,,	200
1	—	0·2	0·6	,,	180 (approx.)
1	—	0·4	1·2	,,	180 ,,
—	—	0·2	0·5	Crystalline	>200 ,,
—	—	0·4	1·0	,,	>200 ,,
1	—	0·2	0·5	,,	200 ,,
1	—	0·4	1·0	,,	180 ,,

Washed heart muscle, either alone or in the presence of lactate alone, coenzyme alone or any of the vitamin preparations alone hardly reduced methylene blue. The addition of crude vitamin B_1 preparations, however, in the presence of lactate caused the reduction of methylene blue in the same way as did the coenzyme prepared from heart muscle. The activity of 0·4 cc. crude vitamin concentrate was equivalent to 0·2 cc. lactic dehydrogenase coenzyme solution. The vitamin preparations which had been further purified by phosphotungstate fractionation had no coenzyme-like activity, which shows that the coenzyme and vitamin cannot be identical.

Test of heart muscle extract for vitamin activity.

The lactic dehydrogenase prepared from heart muscle was tested for its vitamin B_1 content by estimation of its power to promote growth and prevent or cure polyneuritis in rats [Chick and Roscoe, 1929]. These tests were carried out at the Lister Institute, London, through the kindness of Miss M. H. Roscoe. No vitamin B_1 could be detected in doses of 0·1 cc. or 0·2 cc. of the extract, these being insufficient to prevent loss of weight or the development of polyneuritis. Traces of the vitamin were detectable in doses of 2·0 cc. Not sufficient material was available for an accurate test; it could only be stated that 2·0 cc. contained less than one vitamin B_1 unit, or that one pigeon dose was more than 3·0 cc. This material was therefore less than one-tenth as rich in vitamin B_1 as the crude yeast concentrate.

Meiklejohn, Passmore and Peters [1932, 2] have found that the oxygen uptake of avitaminous brain with added vitamin B_1 concentrates was not increased by the addition of pyruvate, while the respiration of normal brain was increased by pyruvate. The system of avitaminous brain *plus* vitamin B_1 concentrate differs from the heart muscle system which oxidises pyruvate readily. The oxidation of pyruvate and of succinate by washed heart muscle occurs without any coenzyme, as is shown by the results in Table II. Pyruvate and succinate are oxidised readily by the preparation, which indicates that pyruvic dehydrogenase and succinic dehydrogenase require no coenzyme or that the coenzyme is not removed by extensive washing.

Table II. *Reduction of methylene blue by washed heart muscle in presence of different substrates.*

2 cc. washed heart muscle suspension in phosphate buffer p_H 7·4 + 1 cc. 0·01 % methylene blue. Substrate concentrations $M/100$.

Substrate	Reduction time in minutes
None	>200
Lactate	>200
Pyruvate	39
Succinate	33
Fumarate	>200
Malate	>200
Malonate	>200
Lactate + succinate	36
Pyruvate + succinate	15

Effect of coenzyme on tissue respiration.

The brain tissue of avitaminous pigeons resembles tumour tissue in respect of being deficient in respiratory power. As the respiration of avitaminous brain

Table III. *Oxygen uptake of thin slices of Jensen rat sarcomata in Ringer lactate solution.*

$mm.^3$ O_2 per g. tissue per hour.

Conditions	First hour	Second hour
Experiment I		
Tissue alone	2200	2200
+ 0·1 cc. coenzyme solution	2130	2300
+ 0·2 cc. coenzyme solution	2200	1850
+ 0·1 cc. vitamin B_1 (purified by phosphotungstate) 0·3 pigeon dose	1950	1850
+ 0·2 cc. vitamin B_1 (purified by phosphotungstate) 0·6 pigeon dose	1860	1920
Experiment II		
Tissue alone	1950	1900
+ 0·1 cc. coenzyme	1970	2000
+ 0·2 cc. coenzyme	2180	1850

in vitro is raised by vitamin B_1, the effect of the vitamin on tumour respiration was investigated. Thin slices of small non-necrotic Jensen rat sarcomata were suspended in 1 cc. Ringer solution containing $M/100$ lactate in the vessels of the Warburg apparatus and the vessels filled with oxygen. The oxygen uptake at 37·5° was measured over 2 hours as shown in Table III.

The experiments show a slightly decreased respiration in the presence of vitamin. In the coenzyme experiments the respiration did not differ significantly from that of the controls. The deficient respiration of the Jensen rat sarcoma is therefore not likely to be due to lack of vitamin B_1 or of lactic dehydrogenase coenzyme.

SUMMARY.

1. Crude vitamin B_1 concentrates resemble Szent-Györgyi's lactic dehydrogenase preparation in enabling washed heart muscle to oxidise lactate. Purified preparations of vitamin B_1 do not behave in this way.
2. Lactic dehydrogenase coenzyme preparations from heart muscle have no vitamin B_1 activity.
3. Washed heart muscle, which oxidises lactate only on the addition of lactic dehydrogenase coenzyme, oxidises pyruvate and succinate without the addition of any coenzyme.
4. The respiration of slices of Jensen rat sarcoma in the presence of lactate was not increased by the addition of lactic dehydrogenase coenzyme or vitamin B_1 solution.

I am deeply indebted to Prof. R. A. Peters and Miss M. H. Roscoe for their valuable help in this work.

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CV. STUDIES IN TISSUE METABOLISM.

II. THE INHIBITION OF LACTIC DEHYDROGENASE BY DERIVATIVES OF CARCINOGENIC COMPOUNDS.

By ERIC BOYLAND.

From the Research Institute, The Cancer Hospital (Free), London.

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During the last few years, work on the chemistry of carcinogenesis has yielded pure hydrocarbons of considerable activity. The first pure hydrocarbon which was found to produce cancer was 1 : 2 : 5 : 6-dibenzanthracene [Cook *et al.*, 1932], and it was then shown that 5 : 6-*cyclo*penteno-1 : 2-benzanthracene [Cook, 1932] and some derivatives of 1 : 2 : 5 : 6-dibenzanthracene were carcinogenic when applied to the skin of mice. Cook, Hewett and Hieger [1933] have described the isolation, identification and synthesis of a very active carcinogenic compound present in coal tar, namely 1 : 2-benzpyrene. This has proved to be the most active carcinogenic substance yet known. Now that such active and well-defined substances which produce cancer are available it was thought that a study of the action of these on enzyme systems might give some indication of the biochemical changes occurring in tumour production.

Cancer is known to be produced not only by coal tar and the hydrocarbons mentioned but also by arsenic [Hutchinson, 1888]. Barry, Bunbury and Kennaway [1928] were able to show that arsenites inhibited xanthine oxidase, the oxidation of acetaldehyde by platinum and the oxidation of propaldehyde and acetaldehyde in the presence of glycine and phosphate. Szent-Györgyi [1930] also found that arsenites inhibited the respiration of liver and of yeast, addition of 0·00013 M arsenite causing 68 % decrease in the respiration of liver pulp. Banga and Szent-Györgyi [1932] have found that the part of the respiratory system of heart muscle which is inhibited by arsenite is concerned with lactate oxidation. They have succeeded in dividing this system into enzyme and coenzyme, and Banga, Szent-Györgyi and Vargha [1932] have described the preparation of this coenzyme. Some experiments recorded below show that arsenite inhibits lactic dehydrogenase, and that derivatives of carcinogenic hydrocarbons, which are chemically quite unrelated to arsenite, have a similar effect on this system. Thus derivatives of two distinct and chemically unrelated types of substance which produce cancer have the same action on this enzyme. The work of Warburg [1926] has shown that malignant tissue differs from most normal tissues in producing more lactic acid than it is able to oxidise, and hence is said to have a large aerobic glycolysis.

The work of Keilin [1929] has shown that the respiratory system of most cells can be divided into three parts: (1) indophenol oxidase, or "Atmungsferment" of Warburg, which activates oxygen; (2) cytochrome, a reversibly reducible pigment which acts as oxygen carrier; (3) the dehydrogenases, *e.g.* lactic dehydrogenase, which activates lactic acid so that it can reduce cytochrome or methylene blue. The respiration of tumours is greatly increased by the addition of *p*-phenylenediamine, showing that there is ample indophenol oxidase or "respiratory enzyme" [Vietorisz, 1931; Boyland and Boyland, unpublished work]. Cytochrome has been found in most malignant tissues examined.

The defect of malignant tissue in lactate oxidation is therefore probably due to deficiency in dehydrogenases. Neither arsenite [Szent-Györgyi, 1930] nor the derivatives of carcinogenic hydrocarbons influence the action of indophenol oxidase, but they are able to inhibit the dehydrogenase systems in which cancer cells are deficient.

No claim is made here that the carcinogenic agents are the only substances which inhibit lactic dehydrogenase. Quastel and Wheatley [1931] found that some dyestuffs had a similar effect. Malachite green and acriflavine in dilutions of 1 in 5000 inhibited 90 % of the respiration of *B. coli* in the presence of lactate. Davies and Quastel [1932] showed that narcotics had a similar action. The results tabulated below show that some non-carcinogenic substances likewise inhibit lactic dehydrogenase.

EXPERIMENTAL.

Polycyclic hydrocarbons are insoluble in water. For experimental purposes solutions in benzene, toluene and *cyclo*hexane were used. Measured amounts of such solutions were shaken with the enzyme preparation in phosphate buffer and one drop of capryl alcohol (to prevent frothing) in ordinary Thunberg tubes. The substrate and finally methylene blue solution were then added and the tube was evacuated. The reduction time at 37° was determined by observation in a glass-sided thermostat.

The preliminary experiments were carried out with 1 : 2 : 5 : 6-dibenzanthracene synthesised by the method described by Clar [1929]. This was dissolved in toluene to give a solution containing 0·1 g. per 100 cc. This was tested with (1) xanthine oxidase from milk, (2) lactic dehydrogenase of yeast, (3) glucose dehydrogenase from liver, (4) citric dehydrogenase from liver, and (5) succinic

Table I. *Effect of 1:2:5:6-dibenzanthracene on oxidising enzymes.*

		Reduction time (in mins.) with		
			1 cc. 0·1 % 1 : 2 : 5 : 6-dibenzanthracene in toluene	
Enzyme preparation	Substrate	1 cc. toluene	Not exposed to sunlight	After exposure to sunlight
Yeast lactic dehydrogenase (described in text, after Bernheim [1928])	Lactate *M*/80	10	10	16
Washed heart muscle, lactic dehydrogenase + coenzyme [Banga and Szent-Györgyi, 1932]	Lactate *M*/80	10	11	15½
Heart muscle, indophenol oxidase [Keilin, 1929]		See text		
Skeletal muscle, succinic dehydrogenase [Clutterbuck, 1928]	*M*/250 sodium succinate	9	9½	15
Liver, glucose dehydrogenase [Harrison, 1931]	*M*/40 glucose	10	10	11
Liver, citric dehydrogenase [Bernheim, 1928]	*M*/40 sodium citrate	10	10½	12
Milk, xanthine dehydrogenase [Dixon and Kodama, 1926]	*M*/8000 hypoxanthine	10	10½	12½

and lactic dehydrogenases of muscle. In each case the control tubes received a volume of toluene equal to that used in the experiment. No effect was obtained with freshly prepared solutions of the hydrocarbon when 2 cc. of enzyme in phosphate buffer, 1 cc. of substrate, 1 cc. of methylene blue (0·02 %), and 1 cc. of toluene solution were used. A solution of 1 : 2 : 5 : 6-dibenzanthracene which had stood in daylight for some days was found however to have a distinct inhibitory effect on lactic dehydrogenase and succinic dehydrogenase, but the other enzymes tested were less affected (Table I).

It is difficult to measure the activity of indophenol oxidase as the enzyme produces a blue colour in the presence of the cell débris, and only qualitative results were obtained. When the effect of irradiated 1 : 2 : 5 : 6-dibenzanthracene was tried on this enzyme derived from yeast cells it was found that the addition of the activated hydrocarbon appeared to increase the rate of production of blue colour from α-naphthol and *p*-phenylenediamine. This shows that the enzyme was not inhibited—the apparent increased activity was probably due to inhibition of dehydrogenases which normally reduce the amount of oxygen available for indophenol formation.

The "activation" of 1 : 2 : 5 : 6-dibenzanthracene was found to occur more rapidly in ultra-violet light. A solution which previously had no effect on lactic dehydrogenase had a marked inhibitory action after 2 hours' irradiation from a mercury arc lamp.

Preparation of lactic dehydrogenase.

For the subsequent experiments lactic dehydrogenase was used as here described. Bernheim [1928] prepared the enzyme from baker's yeast zymin by extraction with alkaline phosphate and dialysing to remove metabolites. The modified method which was used depended upon washing the zymin free from metabolites previous to extraction with phosphate. 10 g. baker's yeast zymin prepared according to details given by Harden [1932][1] were washed 3 times by grinding with water and then centrifuging.

It was then found not to reduce methylene blue without the addition of lactate. The washed zymin was suspended in 100 cc. $M/10$ phosphate buffer p_H 8·4 and allowed to stand overnight between 0° and 5°. The suspension was then centrifuged, the clear liquid which contained lactic dehydrogenase was adjusted to p_H 7·4 and the reduction time of 2 cc. of the enzyme solution with 1 cc. $M/20$ lactate and 1 cc. 1 in 5000 methylene blue at 37·0° measured. The reduction time was generally under 10 minutes, and the enzyme was diluted carefully until the reduction time under these conditions was exactly 10 minutes. In this way a preparation with presumably a constant amount of enzyme is obtained.

In all experiments with inhibitors, the solutions were placed in Thunberg tubes in the following order:

(1) 1 drop capryl alcohol;
(2) inhibitor;
(3) 2 cc. enzyme solution;
(4) 1 cc. $M/20$ lithium lactate;
(5) 1 cc. 1 in 5000 methylene blue.

If lactate is added before the inhibitor less inhibition is produced because the lactate protects the enzyme from inhibition. If methylene blue is added before the lactate greater inhibition occurs, possibly because methylene blue itself destroys lactic dehydrogenase.

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[1] From baker's yeast kindly supplied by The Distillers' Co., Gt Burgh, Epsom.

Solutions in benzene of a number of hydrocarbons[1] which have been tested for carcinogenic power were therefore irradiated and the irradiated solutions tested on lactic dehydrogenase. For irradiation, 10 cc. of a solution (0·01 % in benzene) of each hydrocarbon were placed in a transparent silica tube 10 inches from a vacuum-type vertical mercury vapour lamp[2]. The tubes were left unstoppered open to the air. After 6 hours portions (0·2 cc.) of the solutions were added to 2 cc. of lactic dehydrogenase solution in $M/15$ phosphate buffer, p_H 7·4; lactate and methylene blue were then added, and the reduction time was observed (Table II). The method of indicating carcinogenic power by means of + signs is very rough, but it is extremely difficult to assess this action on a quantitative basis. The signs indicate the relative order of activity of the different compounds.

Table II. *Effect on lactic dehydrogenase of compounds irradiated for 6 hours in benzene solution.*

	Percentage inhibition	Results of test for carcinogenic activity
Benzene (control)	Nil	–
Anthracene	5	–
1 : 2-Benzanthracene	5	+
3 : 4-Benzphenanthrene	13	+ +
1 : 2 : 5 : 6-Dibenzanthracene	26	+ + + +
5 : 6-*cyclo*Penteno-1 : 2-benzanthracene	24	+ + + +
6 : 7-*cyclo*Penteno-1 : 2-benzanthracene	28	+ +
Pyrene	26	+
1 : 2-Benzpyrene	39	+ + + + +
4 : 5-Benzpyrene	20	– (at present)
Chrysene	17	+

Although benzene is a convenient solvent from the point of view of stability and the ease with which the complex hydrocarbons dissolve in it, it has a very wide absorption band in the ultra-violet. For this reason, further experiments were carried out using *cyclo*hexane as solvent. The same technique was employed with a larger range of substance (Table III). The tubes, each containing a different substance in solution, were irradiated in batches of ten at a time. With every batch irradiated in this way a tube containing anthracene was irradiated as control. In all the experiments there was good agreement between different determinations of the change with anthracene. Anthracene in *cyclo*hexane was examined 10 times in this way and the percentage inhibitions produced after 24 hours' irradiation in successive experiments were 50, 42, 45, 54, 52, 48, 43, 40, 46 and 41. The effect of the photochemically oxidised substances on the enzyme was always determined in duplicate and repeated if agreement within 5 % was not obtained. The first part of Table III contains data referring to hydrocarbons which have been tested for carcinogenic activity and found to give negative results. (Some of the mouse-painting tests of compounds occurring in the first part of Table III are not yet complete. The designation of the substances as non-carcinogenic is subject to the possible future

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[1] I am indebted to Dr J. W. Cook for specimens of over 50 of the hydrocarbons used in this work. The syntheses of 33 of these are described in a series of papers by him in the *Journal of the Chemical Society*, 1931 and 1932.

[2] As supplied by the Thermal Syndicate.

Table III. *Percentage inhibition of lactic dehydrogenase produced by 0·2 cc. 0·01 °/₀ cyclohexane solutions of various hydrocarbons irradiated in ultra-violet light.*

	Hours' irradiation			
	0	3	6	24
Non-carcinogenic hydrocarbons:				
Naphthalene	0	7	9	14
Acenaphthene	2	6	—	19
Anthracene	0	10	22	46
Phenanthrene	0	10	12	20
Triphenylene	2	7	11	25
Naphthacene	0	3	15	26
Fluoranthene	0	5	8	10
Retene	0	0	7	10
3′-Methyl-1 : 2-benzanthracene	0	0	6	14
7-Methyl-1 : 2-benzanthracene	0	0	4	11
2′-Methyl-1 : 2-benzanthracene	2	0	12	10
3-Methyl-1 : 2-benzanthracene	0	3	3	5
2′ : 7-Dimethyl-1 : 2-benzanthracene	0	5	2	10
3′ : 6-Dimethyl-1 : 2-benzanthracene	2	3	8	15
3′ : 7-Dimethyl-1 : 2-benzanthracene	0	14	8	13
2′ : 6-Dimethyl-1 : 2-benzanthracene	2	4	8	11
3-*iso*Propyl-1 : 2-benzanthracene	0	0	0	0
7-*iso*Propyl-1 : 2-benzanthracene	2	5	7	17
10-*iso*Propyl-1 : 2-benzanthracene	0	4	0	28
6-Phenyl-1 : 2-benzanthracene	0	4	10	8
10-Benzyl-1 : 2-benzanthracene	0	5	0	12
9 : 10-Diphenyl-1 : 2-benzanthracene	0	2	5	5
Perylene	0	8	15	21
1 : 2 : 3 : 4-Dibenzanthracene	0	0	17	20
1 : 2 : 6 : 7-Dibenzanthracene	0	5	5	16
3 : 4 : 5 : 6-Dibenzphenanthrene	0	7	9	18
1′ : 2′-Naphtha-1 : 2-anthracene	0	10	6	20
2′ : 3′-Naphtha-1 : 2-phenanthrene	2	8	22	24
2′ : 3′-Naphtha-1 : 2-anthracene	0	7	16	25
4 : 5-Benzpyrene	0	6	11	25
7 : 7′-Dimethyl-2′ : 3′-naphtha-1 : 2-anthracene	0	7	12	23
1 : 2 : 3 : 4 : 5 : 6-Tribenzanthracene	0	4	10	16
2′ : 1′-Anthra-1 : 2-anthracene	0	11	15	26
1′ : 2′-Anthra-1 : 2-anthracene	2	8	19	22
1 : 2 : 1′ : 2′-Dibenz-6 : 6′ (or 7 : 7′)-dianthryl	0	5	5	10
Dinaphthachrysene	0	5	8	30
Substances which have produced papillomata only:				
Pyrene	0	16	7	14
Chrysene	0	4	5	9
1 : 2 : 7 : 8-Dibenzanthracene	2	7	9	11
Carcinogenic substances:				
1 : 2-Benzanthracene	0	5	—	21
6-Methyl-1 : 2-benzanthracene	2	6	—	19
6 : 7-Dimethyl-1 : 2-benzanthracene	0	12	—	16
6-*iso*Propyl-1 : 2-benzanthracene	0	4	—	35
1 : 2 : 5 : 6-Dibenzanthracene	0	4	—	36
2′-Methyl-1 : 2 : 5 : 6-dibenzanthracene	0	10	—	48
3′-Methyl-1 : 2 : 5 : 6-dibenzanthracene	0	2	—	28
9-Amino-1 : 2 : 5 : 6-dibenzanthracene	0	0	—	41
9-Methoxy-1 : 2 : 5 : 6-dibenzanthracene	0	6	—	40
9 : 10-Dibenzyl-1 : 2 : 5 : 6-dibenzanthracene	0	4	—	35
5 : 6-*cyclo*Penteno-1 : 2-benzanthracene	0	6	—	35
Phenanthra-acenaphthene	2	6	—	34
1 : 2-Benzpyrene	0	8	—	37
3 : 4-Benzphenanthrene	0	2	—	34
Biological products:				
Ketohydroxyoestrin	0	—	—	36
Cholesterol	0	—	—	32
Ergosterol	0	—	—	20
Oleic acid	0	—	—	34
Glucose	0	—	—	Nil
Hypoxanthine	0	—	—	11
Glutathione	0	—	—	11

production of tumours in the mice under test.) Of these, anthracene produces the greatest inhibitory action. Anthracene irradiated in benzene does not have so large an effect; it is known to polymerise to dianthracene to a greater extent in benzene than in other solvents. The table shows that apart from anthracene 4 only of the 47 non-carcinogenic hydrocarbons in the amounts used (0·2 cc. of 1 in 10,000 solution irradiated for 24 hours) produce more than 25 % inhibition, the maximum figure being 30 % (dinaphthachrysene).

On the other hand, turning to the second part of Table III which refers to known carcinogenic compounds it is seen that most of the carcinogenic substances produce over 30 % inhibition under the same conditions. Thus it can be said that on the whole carcinogenic compounds are more readily changed to give substances which have the power of inhibiting lactic dehydrogenase, but there is no close parallelism. Thus one of the least active cancer-producing compounds (2′-methyl-1:2:5:6-dibenzanthracene, 48 % inhibition) yields more of the inhibitory product than does one of the most active (1:2-benzpyrene, 37 % inhibition).

The concluding section of Table III contains substances of biological interest which were examined in the same way. I am indebted to Dr G. F. Marrian for the sample of ketohydroxyoestrin used in the experiment. It will be noticed that after irradiation both cholesterol and ketohydroxyoestrin produce inhibition of lactic dehydrogenase to an extent comparable with that produced by the carcinogenic substances. It is, however, certain that cholesterol, which is widely distributed in the tissues of normal animals, is not carcinogenic.

The effect of different solvents.

The case of anthracene shows that the reaction which the hydrocarbons undergo is not independent of the solvent used, as anthracene is much more readily changed in *cyclo*hexane than in benzene. In order to study the effect of different solvents, 1 : 2 : 5 : 6-dibenzanthracene was irradiated in the solvents shown in Table IV. The amounts of the various reagents were those stated on p. 793. The most effective solvents are apparently methylethylketone, *p*-xylene and amyl acetate. It is remarkable that there should be such large differences between *o*-, *m*- and *p*-xylenes.

Table IV. *Effect on lactic dehydrogenase of 1:2:5:6-dibenzanthracene irradiated with ultra-violet light for 24 hours in the presence of air.*

1 mg. 1 : 2 : 5 : 6-dibenzanthracene irradiated in 10 cc. solvent.

Solvent	Percentage inhibition by 0·2 cc. solution	Absorption band of solvent between 2000 Å. and 4000 Å. (data from literature)
Methyl alcohol	16	Very slight general absorption
Ethyl alcohol	35	Very slight general absorption
Acetone	37	2300–2950
Methylethylketone	64	2320–2980
Hexane	22	Very slight
*cyclo*Hexane	31	Slight
Benzene	40	2300–2750
Toluene	42	2200–2650
o-Xylene	41	2600–2800
m-Xylene	24	2600–2800
p-Xylene	58	2600–2800 + large band at 2800
Nitrobenzene	13	Very slight
Amyl acetate	56	Large band below 4000
Carbon tetrachloride	28	Weak general absorption

The more effective solvents for the reaction have very marked absorption bands in the ultra-violet. Hexane, methyl alcohol and nitrobenzene, which are very transparent to ultra-violet light between 2000 Å. and 3000 Å., give very small amounts of "activated" hydrocarbon; in this respect ethyl alcohol is an exception. The difference in absorption spectra between *p*-xylene on the one hand and *o*- and *m*-xylene on the other is considerable [Baly and Tryhorn, 1915], in that *p*-xylene has a very strong band at 2800 Å., while the *o*- and *m*- isomerides have no such bands. Similarly methylethylketone absorbs more between 2200 Å. and 3000 Å. than does acetone [Bielecki and Henri, 1912]. The absorption bands of polycyclic hydrocarbons also occur between 2000 Å. and 3000 Å., so that it might be expected that the solvents which absorb ultra-violet light of that wave length would hinder the reaction, but this is not so.

Regener [1906] showed that ozone was decomposed by light of 2000–3000 Å. and Schumann [1903] found that oxygen was converted into ozone by light of shorter wave-length than 1950 Å. Experiments described below show that oxygen is necessary for the "activation" of hydrocarbons, and it is possible that the rôle of ultra-violet light is to convert O_2 into O_3, which might combine with the hydrocarbons to form an ozonide or peroxide. Such an explanation would be in agreement with the results obtained on comparing the effect of different solvents.

Nature of the active agent.

The preparation of aqueous colloidal suspensions of 1 : 2 : 5 : 6-dibenzanthracene has been described elsewhere [Boyland, 1932]. Such solutions were found to be completely inactive towards lactic dehydrogenase even after long exposure to daylight. Under such conditions the hydrocarbon could come into perfect physical contact with the enzyme, and hence the result shows that on irradiation some chemical change must occur. After irradiation in benzene or *cyclo*hexane the solutions of carcinogenic hydrocarbons lose fluorescence and become yellow or slightly brown.

Solutions of 1 : 2 : 5 : 6-dibenzanthracene and 1 : 2-benzpyrene in benzene were boiled to remove dissolved oxygen and then irradiated in closed quartz tubes in the presence of nitrogen for 24 hours. The solutions were then colourless and strongly fluorescent, while control solutions which had been irradiated while exposed to the air were yellow and less fluorescent. The solutions irradiated anaerobically were free from inhibitory action towards lactic dehydrogenase, while those irradiated in contact with air were actively inhibitory. This indicates that the active inhibitory substances are oxidation products.

The fact that these substances inhibit water-soluble enzymes suggests that the product is water-soluble. To test this, a benzene solution of 1 : 2 : 5 : 6-dibenzanthracene which had been oxidised in air in ultra-violet light was extracted with aqueous solutions of different p_H, the extracts were neutralised and tested with lactic dehydrogenase. The results are given in Table V and Fig. 1. The active extracts made with alkaline solutions were brown in colour and the depth of colour appeared to follow the degree of activity.

Carcinogenic hydrocarbons are therefore capable of oxidation in ultra-violet light to acidic substances, which can inhibit dehydrogenating enzymes. Although the substances react as acids in their solubility relationships it is not certain that they are carboxylic acids. Qualitative experiments showed that the oxidised product could liberate iodine from hydriodic acid in glacial acetic acid solution.

Table V. *Effect of* p_H *on the amount of inhibitor extracted from a photochemically oxidised solution of 1:2:5:6-dibenzanthracene in benzene.*

Solution used for extraction	p_H of solution	Percentage inhibition produced by 0·5 cc.
Water	6	10
N/10 acetic acid	3	10
M/10 KH_2PO_4	5	24
M/10 phosphate p_H 7·0	7	28
M/10 Na_2HPO_4	8	35
M/10 K_2CO_3	10	49
N/100 NaOH	12	58

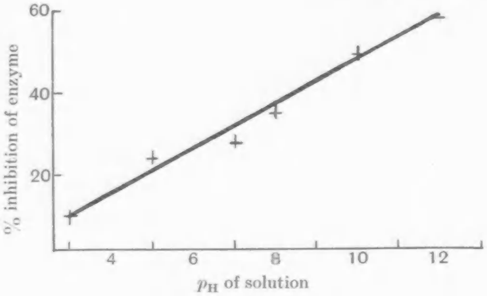

Fig. 1 (see Table V).

Hartmann and Siebert [1932] and Hock and Sosemihl [1933] have described the isolation of tetrahydronaphthalene peroxide produced by oxidation of tetrahydronaphthalene with air, and this compound forms a water-soluble sodium salt, while the free peroxide is insoluble in water. It is conceivable that the oxidation of carcinogenic hydrocarbons yields a peroxide and that it is this compound which is able to destroy dehydrogenating enzymes. So far all attempts to isolate the pure inhibitory compound produced by oxidation of 1 : 2 : 5 : 6-dibenzanthracene have been unsuccessful, although qualitative evidence for the production of 1 : 2 : 5 : 6-dibenz-9 : 10-anthraquinone and a dicarboxylic acid has been obtained. That the inhibiting substance is not a dicarboxylic acid produced by opening of one of the rings is made probable by the fact that phthalic acid and naphthalene-1 : 2-dicarboxylic acid have no inhibitory power, and more complex dicarboxylic acids such as deoxybilianic acid and *iso*deoxybilianic acid show but slight activity. The active product is not so stable in aqueous solution as would be expected of a dicarboxylic acid.

As it was known that the active inhibitory principle produced from the hydrocarbons was alkali-soluble, solutions of 1 : 2 : 5 : 6-dibenzanthracene in benzene and toluene were exposed to ultra-violet light while being incubated over dilute sodium carbonate solution. The solutions were irradiated in quartz tubes and in some experiments the lower aqueous layer was shaded from the ultra-violet light, so that the alkali-soluble product would be less liable to change. In all experiments of this nature in which water or alkaline solutions were left in contact with the hydrocarbon solution, much smaller yields of inhibiting substance were obtained. The presence of water appeared to interfere with the oxidation process. This again suggests that the action is due to ozone, for it is well known that water tends to decompose ozone.

Effect of ozone on hydrocarbons.

Ozone was made by passing oxygen through a tube subjected to a silent electrical discharge. The ozonised oxygen was passed through the liquid under treatment at a constant rate. Solutions treated with ozone in this way were then tested for inhibitory action on lactic dehydrogenase. Controls were carried out by treating the solvent used with ozone for the same time as the solution. *cyclo*Hexane was found to be apparently unaffected by ozone when treated in this way, but solutions of 1 : 2-benzpyrene in benzene or *cyclo*hexane quickly changed, becoming yellow-coloured and practically non-fluorescent. Similar solutions of 1 : 2 : 5 : 6-dibenzanthracene did not change so rapidly, but even in this case after passing ozone for 3 hours the dibenzanthracene became oxidised and then caused inhibition of lactic dehydrogenase to the extent shown in Table VI.

Table VI. *Effect on lactic dehydrogenase of 1:2-benzpyrene and 1:2:5:6-dibenzanthracene in benzene oxidised with ozone.*

	Reduction time mins.	Percentage inhibition
0·4 cc. benzene	10	—
0·1 cc. 0·1 % 1 : 2-benzpyrene	10½	5
0·2 ,,	15	33
0·4 ,,	16½	40
0·8 ,,	22	55
0·1 cc. 0·1 % 1 : 2 : 5 : 6-dibenzanthracene	10	Nil
0·2 ,,	11½	13
0·4 ,,	12	17
0·8 ,,	15½	36

There is no doubt that ozone is able to oxidise these two carcinogenic hydrocarbons. Possibly an ozonide resembling the anthracene ozonide of Molinari [1907] is formed, but this may in turn change to some other oxidation product.

Comparison of effect with that of arsenite.

The inhibitory effect of arsenite on oxidations has been known since the work of Barry, Bunbury and Kennaway [1928]. The effects of arsenite and oxidised 1 : 2 : 5 : 6-dibenzanthracene were therefore compared quantitatively. The same standard yeast lactic dehydrogenase was used with the concentrations of sodium arsenite shown in Table VII.

Table VII. *Effect of sodium arsenite on the action of dehydrogenase in presence of* $M/80$ *lactate.*

Concentration of $NaAsO_2$	Reduction time mins.	Percentage inhibition
Nil	10	—
$M/660$	10½	5
$M/330$	13	23
$M/180$	15½	36
$M/90$	20	50

Addition of $M/90$ arsenite thus appears to inactivate half the enzyme when the lactate is present in the concentration shown. Table IV shows that 0·2 cc. of 0·01 % 1 : 2 : 5 : 6-dibenzanthracene irradiated for 24 hours in methylethylketone, *p*-xylene or amyl acetate causes more than 50 % inhibition. This is a final concentration equivalent to 0·005 % of the original hydrocarbon or

0·00018 *M*. Thus a concentration approximately only one-sixtieth of that necessary with arsenite inactivates one-half of the enzyme. Activated 1 : 2 : 5 : 6-dibenzanthracene is therefore 60 times as active an inhibitor as arsenite when compared in presence of *M*/80 lactate.

Arsenite is a competitive inhibitor [*cf.* Haldane, 1930] as is shown by the results in Table VIII.

Table VIII. *Effect of lactate concentration on inhibition by arsenite.*

Concentration of lactate	Reduction time (mins.) Without arsenite	Reduction time (mins.) With *M*/60 $NaAsO_2$	Percentage inhibition by arsenite
M/10	9	18½	52
M/20	9½	25	68
M/40	10½	45	79
M/80	12	80	84
M/160	15	120	97

Results of a similar experiment using oxidised 1 : 2 : 5 : 6-dibenzanthracene are shown below (Table IX).

Table IX. *Effect of lactate concentration on inhibition by oxidised 1:2:5:6-dibenzanthracene.*

Concentration of lactate	Reduction time without oxidised 1 : 2 : 5 : 6-dibenzanthracene mins.	Reduction time with 0·2 cc. 0·01 % oxidised 1 : 2 : 5 : 6-dibenzanthracene in benzene mins.	Percentage inhibition
M/4	6¾	14½	53
M/8	6	11	48
M/16	5	11	55
M/25	5½	13½	59
M/50	6	15	59
M/100	6½	17½	62
M/200	8	35	78

The inhibition does not vary much with change of substrate concentration and would therefore appear to be of a non-competitive type. The oxidised hydrocarbon destroys or inactivates a definite amount of enzyme and therefore differs in its effect from that of arsenite which competes with the substrate.

DISCUSSION.

The results in Tables I and II seemed to show that carcinogenic hydrocarbons could be photochemically oxidised to products which destroy dehydrogenating enzymes. In these preliminary experiments the effect seemed to run parallel with the carcinogenic activity of the substance when the oxidation was carried out with only a few substances in benzene solution. It might therefore have been a useful quick test of carcinogenic power, but further work on over 50 substances in *cyclo*hexane showed the parallelism to be very rough, as anthracene and cholesterol, which are non-carcinogenic, were oxidised to powerful inhibitory products.

It is possible that this effect on dehydrogenases might explain the mechanism of cancer production by carcinogenic hydrocarbons. If the dehydrogenating enzymes in the tissues were destroyed there would be a tendency to local accumulation of lactic acid. Tumour cells survive and grow in media containing lactic acid and are probably less affected by it than normal cells. If in tissue in which dehydrogenases were destroyed a malignant cell were to appear it would be at a great advantage and would grow at the expense of normal cells. It is possible that the active oxidation product of the hydrocarbons studied is of a peroxide nature which destroys the enzyme by oxidation. Such an oxidation to a peroxide may occur in the body and the oxidative destruction of the enzyme by this might yield a product capable of reoxidation to an active product. By this means a small quantity of hydrocarbon may inactivate many molecules of enzyme in the body. The possible biological oxidation of carcinogenic hydrocarbons will be studied in the near future.

SUMMARY.

Many polycyclic hydrocarbons are oxidised photochemically in air to products which are able to destroy dehydrogenating enzymes. On the whole, carcinogenic hydrocarbons, such as 1 : 2 : 5 : 6-dibenzanthracene and 1 : 2-benzpyrene, after oxidation are capable of greater inhibition of lactic dehydrogenase than are the non-carcinogenic hydrocarbons.

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CVI. A NOTE ON GLUTATHIONE AND VITAMIN C IN TUMOUR TISSUE.

By ERIC BOYLAND.

From the Research Institute of the Cancer Hospital (Free), London.

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Vitamin C and the hexuronic acid of suprarenal tissue (ascorbic acid) were found to be identical by Svirbely and Szent-Györgyi [1932], and the identity has been confirmed by many other workers. This has led to the chemical estimation of vitamin C by Birch, Harris and Ray [1933]. Birch and Dann [1933] have pointed out that determinations of glutathione in tissues by the method of iodine titration are probably too high because in such a titration iodine would be reduced by ascorbic acid as well as by sulphydryl groups. Thus in an acid extract of suprarenal cortex as much as 77 % of the total iodine required may react with ascorbic acid and only 23 % with glutathione. Almost simultaneously with the publication of this suggestion Bierich and Rosenbohm [1933] produced evidence showing that the figures normally given for the glutathione content of tissues as obtained by iodine titration were probably too high and that some other reducing substance, "*x*," was present. This conclusion was arrived at by comparison of results obtained by iodine titration and a colorimetric method of estimating glutathione. These authors suggest in a footnote that the reducing substance may be the hexuronic acid of Szent-Györgyi. The conclusions of Birch and Dann and of Bierich and Rosenbohm have been confirmed in this laboratory and similar estimations have been carried out upon tumours.

Experimental.

Tissues were removed from animals immediately after killing, rapidly weighed and ground with 3 times their weight of ice-cold 10 % trichloroacetic acid. After standing 30 minutes in the cold the tissue suspension was centrifuged and the extract filtered. The tumours examined were carcinomata (C 63) of mice and sarcomata ($\mathrm{LM}\frac{157}{23\,\mathrm{A}}$, $\frac{4}{59\,\mathrm{E}}$, $\frac{\mathrm{LR}\ 10}{35\,\mathrm{F}}$ and Baker No. 2) of mice, rats and fowls. The designation $\mathrm{LM}\frac{157}{23\,\mathrm{A}}$ is applied to a mouse tumour produced originally by the subcutaneous injection of 5 : 6-*cyclo*penteno-1 : 2-benzanthracene in lard and grafted through 23 generations[1]. Similarly, $\frac{\mathrm{LR}\ 10}{35\,\mathrm{F}}$ refers to the 35th grafted generation of a rat tumour produced by the subcutaneous injection of 1:2:5:6-dibenzanthracene dissolved in lard, and $\frac{4}{59\,\mathrm{E}}$ to the 59th generation of a mouse tumour produced in a similar manner. I am indebted to Prof. McIntosh of the Middlesex Hospital for the fowl tumour (Baker No. 2).

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[1] A description of this tumour will be published by Dr J. W. Cook and Miss Barry.

Ascorbic acid was estimated by titration against phenol-2 : 6-dichloroindophenol as described by Birch, Harris and Ray [1933]; Dr Harris was good enough to furnish full details of the method. In the absence of a specimen of pure ascorbic acid as standard the dye solution was titrated with filtered orange juice. Orange juice was found to contain no glutathione as estimated by the colorimetric method. The ascorbic acid content of the orange juice (and the iodine equivalent of the 2 : 6-dichloroindophenol solution) were therefore estimated by titration with $N/100$ iodine in the presence of 5 % trichloroacetic acid.

Glutathione was estimated colorimetrically by a method similar to that of Bierich and Rosenbohm except that it was carried out on the CCl_3COOH extract, and the colour produced was compared with that produced by glutathione in standard controls containing 0·5, 1·0, 1·5 and 2·0 mg. glutathione. The neutralised CCl_3COOH extract was saturated with $(NH_4)_2SO_4$, and volumes ranging from 2 to 12 cc. of the solution were transferred with a pipette into a 15 cc. graduated flask; 1·0 cc. M KCN and 1·0 cc. 1 % sodium nitroprusside were added and then saturated $(NH_4)_2SO_4$ solution to 15 cc. The reddish-purple colour produced was rapidly compared in a colorimeter with that produced at the same time by standard glutathione solution.

The method is very crude as the colour produced by glutathione fades rapidly and is not exactly proportional to the amount of this compound. By taking an amount of standard solution such that the control and the experiment contain about the same amount of glutathione, duplicate results are obtained which do not differ by more than 10 %.

The iodine titration which presumably gives a measure of ascorbic acid *plus* glutathione was carried out by the method of Tunnicliffe [1925].

Table I shows the result of ascorbic acid determinations on some rat and mouse tissues. The figures obtained by Birch and Harris and by Bierich and

Table I. *Ascorbic acid content of normal and malignant tissues.*

	Ascorbic acid			
Tissue	Estimated with indophenol mg. per g.	Calculated equivalent $N/100$ I per g. Average cc. per g. tissue	From Birch and Dann. Equivalent $N/100$ I cc. per g. tissue	From Bierich and Rosenbohm. Substance "x," equivalent $N/100$ I cc. per g. tissue
Mouse				
Muscle	0·04, 0·03, 0·05	0·048	—	—
Liver	0·25, 0·31, 0·26	0·31	—	—
Sarcoma				
LM $\frac{157}{23\ A}$	0·16, 0·11, 0·15	0·16	—	—
$\frac{4}{59\ E}$	0·20, 0·16	0·20	—	—
Rat				
Blood	0·05, 0·03	0·04	0·023	—
Muscle	0·04, 0·04, 0·05	0·05	0·068	0·03–0·04
Liver	0·32, 0·34, 0·36	0·39	0·188	0·07–0·56
Kidney	0·26, 0·27	0·30	0·25	0·60–1·2
Spleen	0·22	0·25	—	0·22–0·47
Brain	0·26	0·30	—	0·33–0·48
Jensen rat sarcoma	0·50, 0·48, 0·47, 0·45	0·54	—	—
Sarcoma $\frac{LR\ 10}{35\ F}$	0·22, 0·27	0·28	—	—

Rosenbohm are given for comparison. The ascorbic acid values are of the same order as those of Bierich and Rosenbohm for the unknown reducing substance "*x*." It seems highly probable that the reducing substance "*x*" is identical with ascorbic acid.

The estimations on muscle and blood are less accurate than the others because of the small amounts of both ascorbic acid and glutathione present in these. Results obtained with kidney tissue are also open to suspicion on account of the possible presence of cysteine.

Table II. *Ascorbic acid and glutathione.*

		1	2	3	4	5	6	7
		Ascorbic acid from phenol-2 : 6-dichloro-indophenol titration		Glutathione determined colorimetrically		Total I required Tunnicliffe method	I equivalent of ascorbic acid + glutathione	Percentage of total I (col. 5) due to GSH
Tissue		mg. per g.	Equivalent cc. *N*/100 I	mg. per g.	Equivalent cc. *N*/100 I	cc. *N*/100 I	(col. 2 + col. 4)	(col. 4)
Mouse								
Liver	(1)	0·28	0·32	3·50	1·14	1·35	1·46	84
	(2)	0·26	0·30	3·30	1·08	1·15	1·38	93
Muscle	(1)	0·045	0·05	0·31	0·11	0·23	0·16	48
	(2)	0·052	0·06	0·25	0·08	0·11	0·14	73
Carcinoma	(1)	0·105	0·12	0·21	0·07	0·19	0·19	37
C 63	(2)	0·115	0·13	0·18	0·06	0·21	0·19	29
	(3)	0·13	0·15	0·25	0·08	0·19	0·23	42
Rat								
Liver	(1)	0·26	0·29	1·45	0·47	0·72	0·76	65
	(2)	0·25	0·28	1·54	0·50	0·73	0·78	68
	(3)	0·23	0·26	1·54	0·50	0·70	0·76	71
Kidney	(1)	0·35	0·40	0·86	0·28	0·63	0·68	45
	(2)	0·38	0·43	0·92	0·30	0·72	0·73	42
Spleen	(1)	0·18	0·20	0·89	0·29	0·45	0·49	64
	(2)	0·19	0·21	0·80	0·26	0·53	0·47	49
Muscle	(1)	0·03	0·035	0·14	0·045	0·07	0·08	64
	(2)	0·04	0·05	0·12	0·04	0·06	0·09	66
Brain	(1)	0·22	0·25	0·40	0·13	0·40	0·38	33
	(2)	0·28	0·32	0·68	0·22	0·53	0·54	42
Jensen rat sarcoma	(1)	0·38	0·43	0·55	0·18	0·67	0·61	27
	(2)	0·27	0·31	0·52	0·17	0·47	0·48	36
	(3)	0·42	0·47	0·65	0·21	0·69	0·68	30
Sarcoma $\frac{\text{LR 10}}{\text{35 F}}$	(1)	0·23	0·26	0·37	0·12	0·34	0·38	35
	(2)	0·17 (necrotic)	0·18	—	Nil	0·20	0·18	—
Fowl								
Liver		0·27	0·31	1·35	0·44	0·70	0·71	63
Kidney		0·16	0·18	1·20	0·39	0·63	0·57	62
Muscle		0·04	0·04	0·29	0·10	0·16	0·14	62
Sarcoma (Baker No. 2)		0·11	0·13	0·38	0·12	0·28	0·25	43

The results shown in Table II indicate that the method of iodine titration of acid extracts of tissues using starch as indicator gives a value which includes both ascorbic acid and glutathione, and that in some tissues the values that have been assumed for glutathione content are in reality too high. The amount of iodine required by the tissue extracts (column 5) agrees fairly closely with the theoretical value of the sum of iodine equivalents of ascorbic acid and glutathione present (column 6).

It was first stated that tumours contained no glutathione [Holmes, 1926], but Bierich and Kalle [1926], Thomson and Voegtlin [1926] and Kennaway and Hieger [1927] all found glutathione in malignant tissues when the iodine titration method was employed; Yaoi and Nakahara [1926] and Bierich and Rosenbohm [1933] have found it by colorimetric (nitroprusside) methods. The results shown in Table II indicate that the values which have previously been given by the iodine titration method are possibly as much as three times too high. The determination of ascorbic acid indicates that the large discrepancy between colorimetric and iodimetric estimations is mainly due to the occurrence of this substance in malignant tissues. Brain tissue resembles tumour tissue and the suprarenal gland in having a high ascorbic acid content compared with the glutathione content.

Summary.

The estimation of glutathione in tumour tissue (sarcoma of rat, mouse, and fowl, carcinoma of mouse) by a colorimetric method gives values only one-third as great as those obtained by iodine titration. Estimations of ascorbic acid show that the high iodine titre is due to the occurrence of this substance in tumour tissue.

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Note added June 19th, 1933. Biological tests on Jensen rat sarcomata carried out by Dr L. J. Harris indicate that the substance in tumours which reduces 2:6-dichlorophenolindophenol, and is designated in this paper as ascorbic acid, is not ascorbic acid. Malignant tissue must therefore contain considerable quantities of some unidentified reducing substance.

CVII. STUDIES ON THE CARBOHYDRATE METABOLISM OF THE CHICK EMBRYO.

By CONSTANTIN DONHOFFER.

From the Medical Clinic of the University, Peés, Hungary.

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PART I. THE CARBOHYDRATE CONTENT OF THE EGG.

Since the avian egg represents, in a certain sense, a closed system, changes of its carbohydrates during development may lead to the disclosure of some facts concerning the intermediary metabolism of these substances which are not readily revealed under other conditions. As the first step in the investigation of these changes, suitable methods for the differentiation of the various intermediary substances had to be selected, and it is with this problem that the first part of this paper is concerned. Methods used by previous investigators of the problem have recently been critically reviewed by Needham [1927; 1931], but it cannot be admitted that even his procedure is free from possible sources of error. This is particularly the case when data taken from "smoothed curves" and from the published results of other workers are used in calculating total carbohydrate.

The figures given by Needham and others for free sugar vary a great deal. This seems to be mainly due to the presence of a water-soluble substance of considerable reducing power, which is not removable with most protein precipitants. The reducing power of alcoholic extracts represents more closely free sugar, but even these show some non-sugar reduction, depending in amount on the exact method used. The differences are necessarily greater when the estimation involves hydrolysis, in which case only a fraction of the observed reduction may be due to carbohydrates. In order to reduce these sources of error, in the present investigation, fermentation and precipitation with copper-lime were used for determination of the free sugar, although it is realised that even by these methods substances other than carbohydrate may be included.

Methods.

Eggs of various origin and age were macerated for several hours without their shells in 500 cc. of 96 % alcohol and, after removing the alcohol by centrifuging, extraction was repeated twice with 300 and 150 cc. alcohol respectively. The combined extracts were evaporated (under reduced pressure) at 55–65° and, towards the end of evaporation, several 15–25 cc. portions of distilled water were added. After the evaporation of all the alcohol the residue was taken up in a definite volume of water (usually 100 cc.). The resultant emulsion was precipitated with trichloroacetic acid or phosphotungstic acid, the neutralised filtrate being referred to as the "alcohol-soluble fraction."

The alcohol precipitate was extracted for several hours first with 100 cc., and then twice with 50 cc. of boiling distilled water. The combined extracts, containing the water-soluble fraction of the alcohol precipitate along with the water pressed out from the water-insoluble residue, were then analysed in the various ways described later. The residue, freed from water, was weighed and suspended in an equal volume of water to which HCl was added, to make the concentration 5 %, after which it was hydrolysed on a boiling water-bath. Proteins were then precipitated with phosphotungstic acid, and the resultant solution was worked up in the same way as in all other fractions.

In all extracts, reducing power was estimated, after neutralisation to phenol red, by the Hagedorn-Jensen method (total reduction), and this was repeated in the same solutions after fermentation and after precipitation with copper-lime. The difference between total reduction and that observed after fermentation and precipitation with copper-lime is referred to as "fermentable" and "precipitable" reduction respectively.

The excess of phosphotungstic acid was removed by adding concentrated potassium chloride. Filtration was avoided throughout by centrifuging.

Glycogen was estimated by Pflüger's method, with the difference that fermentable reduction alone was finally measured.

Unless otherwise stated reducing power is expressed throughout in mg. glucose per 50 g. of eggs without shell.

Table I shows decisively that the precipitable reduction of the alcohol-soluble fraction is fermentable *in toto*, supporting the assumption that all this reduction is due to monosaccharides. The figures, which average 108 mg., agree remarkably well in eggs of various origin and laid at different seasons, yet they are considerably lower than those of other workers, which vary between 156 mg. [Pennington *et al.*, 1915] and 225 mg. [Hepburn and St John, 1921]. Only

Table I. *Alcohol-soluble fraction.*

		Reduction (mg. glucose per 50 g. egg)		
Exp.	Total	Precipitable	Fermentable	
1	137	126	—	Single eggs of various origin
2	122	106	—	
3	107	94	—	
4	96	96	96	
5	152	129	122	
6	133	122	114	
7	123	108	111	
8	130	95	95	
9	128	121	121	
10	128	111	111	Average of two eggs
11	115	103	107	
12	131	117	118	
13	135	117	114	
14	112	94	96	Fresh Rhode Island eggs
15	109	97	—	
16	106	95	—	
17	102	92	90	
18	130	113	110	
19	124	106	—	
20	120	103	105	
21	129	107	—	
22	131	114	112	

Müller and Masayama's [1900] are lower (40–97 mg.). This divergence is probably mainly due to methodical errors.

In some of these experiments reducing power was estimated after hydrolysis as well (2·2 % HCl, 1·5–2·5 hours on the boiling water-bath), with the unexpected result, that a regular decrease was observed. The reducing power of glucose does not diminish in similar circumstances, so that the presence of another monosaccharide besides glucose is probable.

Table II. *Alcohol-soluble fraction. Loss in reducing power after hydrolysis. (mg. glucose per 100 mg. originally present).*

		Reduction loss	
Substance	Duration of hydrolysis hours	Precipitable	Fermentable
Whole egg	2·5	20	—
,,	2·5	13	16
,,	1·5	3	5
,,	2·5	24	18
,,	2·0	0	0
,,	2·0	8	10
2 Whites	2·5	11	13
2 Yolks	2·5	13	8
Whole egg	2·0	9	12
,,	2·0	16	—
,,	2·0	13	—
,,	2·0	13	11

Table III shows that this decrease in reducing power is continuous during a certain period.

Table III. *Alcohol-soluble fraction of egg-white. Loss in reducing power after hydrolysis with 2·2 °/₀ HCl.*

Before hydrolysis	Reduction 208 mg./100 cc.
15 mins. at room temperature	202
30 mins. at 100°	196
60 mins. at 100°	192
90 mins. at 100°	186
180 mins. at 100°	173

The water-soluble fraction of the alcohol precipitate. After clearing this fraction of the supernatant fat in a separating funnel, it was hydrolysed with 2·2 % HCl for 2·5 hours in a boiling water-bath, and subsequently the proteins were precipitated with phosphotungstic acid. In other experiments parts of this fraction were precipitated before hydrolysis: (*a*) with phosphotungstic acid, (*b*) with trichloroacetic acid. The resulting solutions and the trichloroacetic acid precipitates were then hydrolysed in a similar way. The precipitable reduction of the trichloroacetic acid filtrate was considerable even without hydrolysis, but none of this was fermentable. In Tables IV, V and VI only the figures referring to reduction after hydrolysis are recorded.

Changes in the duration of hydrolysis, in the concentration of the acid or in precipitant have no effect on the precipitable or fermentable reduction, though all of them alter total reduction.

In this way the water-soluble fraction of the alcohol precipitate can be divided into three fractions: (*a*) not precipitable with phosphotungstic acid, (*b*) precipitable with phosphotungstic acid but not with trichloroacetic acid, (*c*) precipitable with trichloroacetic acid. Table IV shows the behaviour of these fractions. Fraction (*b*) is the difference of the trichloroacetic acid filtrate and fraction (*a*).

Table IV. *Reduction precipitable with copper-lime.*

(January to March.)

	Fraction		
Exp.	(*a*)	(*b*)	(*c*)
1	17	45	22
2	18	52	20
3	16	42	10
4	18	44	8
5	19	45	20
6	16	57	16
Average	17·3	47·5	16·0

Fraction (*a*) gives no Molisch test, is not fermentable and is dialysable even before hydrolysis. Fraction (*b*) is not dialysable before hydrolysis, gives a strong Molisch test and after hydrolysis about 68 % of it is fermentable. Fraction (*c*) gives also a strong Molisch test and after hydrolysis is fermentable *in toto*.

Table V shows the figures of the total precipitable reduction of the water-soluble fraction of the alcohol precipitate during the months December-March and May-August respectively.

Table V.

Dec.-March	87	77	72	74	96	74			
	93	75	77	83	92	89			
	92	77	82	74	89	81			
May-August	66	52	59	46	45	47	54	53	48
	65	42	53	50	56	60	46	54	55
	62	50	52	44	56	51	46	59	58

The water-insoluble fraction of the alcohol precipitate. This fraction was hydrolysed with 5 % HCl for 5–10 hours and afterwards precipitated with phosphotungstic acid. The fermentable reduction is not affected by these variations in the duration of hydrolysis. Precipitation with copper-lime is not satisfactory in this fraction because of difficulty in reading the end-point of titration. So, besides total reduction only the fermentable reduction is recorded, as shown in Table VI.

Table VI.

	January–March				July		
Exp.	(*a*)	(*b*)	(*c*)	(*d*)	(*e*)	(*f*)	(*g*)
Total reduction	206	257	230	244	226	268	257
Fermentable reduction	94	101	93	93	86	77	79

Glycogen per egg is shown in Table VII.

Table VII.

Glycogen per egg.

Exp.	1	2	3	4	5	6	7	8	9	10
Mg.	9·6	9·0	8·2	8·4	7·6	8·0	7·2	11·9	10·0	10·6

The distribution of reducing substances between white and yolk[1] is shown in Table VIII.

Table VIII.

	Mg. glucose per 100 g. egg		Weight of yolk	Reduction of yolk
			In % of whole egg	
Exp.	White	Yolk		
Alcohol-soluble fraction:				
I	312	162	44·3	28·5
II	342	122	41·5	20·2
III	348	158	39·0	22·4
IV	312	148	37·0	22·5
V	318	152	38·0	22·3
Average	326	148	39·9	23·1
Water-soluble fraction of alcohol precipitate:				
I	296	92	44·3	19·6
II	324	83	41·5	15·4
III	222	58	39·0	14·8
IV	229	75	37·0	16·7
V	245	58	38·0	13·0
Average	263	73	39·9	15·9
VI	0·0	16·0		
VII	0·0	15·2		

The result that the yolk contains about 23 % of the total glucose is in agreement with those of Sato [1916], Pennington *et al.* [1915] and Kojo [1911], though their absolute figures are much higher for both white and yolk, being 440–550 and 180–272 mg. per 100 g. respectively. The ratios given by Sakuragi [1917] and Roger [1908] are somewhat higher (30–31 %), due to a greater reduction of the yolk (300–330 mg. per 100 g.). The probable cause of these higher values has been pointed out previously. Tomita [1921] found a lower ratio for the yolk (15 %) combined with low absolute values in both white (84 mg. per 100 g.) and yolk (15 mg. per 100 g.). Figures for the water-soluble fraction of the alcohol precipitate show that the yolk contributes to this portion an even lower percentage of reduction (15·9 %). Contrary to Sakuragi [1917] no glycogen could be found in the white. In respect of hydrolysis and fermentation the yolk and the white behave in the same way.

DISCUSSION.

Regarding the alcohol-soluble fraction, it is of interest that several investigators have found fructose in the allantoic fluid and foetal blood of various species. This makes the assumption probable that the decrease of reduction observed after hydrolysis may be due to destruction of fructose. The question whether alcohol-soluble higher carbohydrates are present, cannot be answered definitely, since an increase of reducing power due to such could be masked by the presence of the above-mentioned reducing substance sensitive to mineral acids.

Of the three fractions into which the water-soluble fraction of the alcohol precipitate can be subdivided, the nature of fraction (*a*) (not precipitable with phosphotungstic acid) is questionable, and fraction (*b*) probably contains all the ovomucoid sugar, the difference between precipitable and fermentable reduction being possibly due to glucosamine hydrochloride. Fraction (*c*) (precipitable with trichloroacetic acid and fermentable *in toto*) represents protein sugar. The same can be said of the fermentable reduction of the water-insoluble fraction of the alcohol precipitate.

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[1] All figures represent the mean of two whites or two yolks.

Glycogen. Direct estimations show that a part of the glycogen is found in the water-soluble, another part in the water-insoluble fraction of the alcohol precipitate. This fact does not interfere with the figures given above, as the amount of glycogen present is small, but must be taken into consideration in experiments done during incubation, when glycogen is accumulating.

The separation into fractions by solubility and precipitation does not offer information on the chemical nature of the carbohydrates involved; it only differentiates substances containing carbohydrates, though differences in fermentability indicate that the carbohydrate component is not identical in all of them. So the question, whether the carbohydrates of the water-soluble, or water-insoluble fraction of the alcohol precipitate or both of them are identical with Levene and Mori's [1929] polysaccharide, containing a trisaccharide composed of two molecules of mannose and one molecule of glucosamine, or with Pringsheim's [1923] or Lohmann's [1926] amylotriose, cannot be answered. Nevertheless, as the figures of these fractions are fairly constant, the differentiation between them seems useful for observations on the carbohydrate metabolism of the developing egg.

The total carbohydrate content of the egg varies in the present experiments between 272 and 296 mg. These figures are considerably lower than those found by other investigators, which vary between 335 mg. [Needham, 1927] and 507 mg. [Sakuragi, 1917]. The methods applied account for this difference. The figures of Pucher [1928] for total fermentable sugar are the only ones which agree with the present results. He found 265 mg. of fermentable sugar present in the egg, and when one considers only the fermentable part of the water-soluble fraction of the alcohol precipitate in the present experiments exactly the same figures are obtained.

PART II. CHANGES DURING INCUBATION.

Using the methods of analysis described in Part I the present work was started to ascertain (*a*) the source of the glycogen formed during incubation, (*b*) the rôle of gluconeogenesis, and (*c*) the nature of the carbohydrate fraction primarily formed by gluconeogenesis, if such occurs. Results on the carbohydrate metabolism during incubation obtained previously differ widely, and the methods applied involve considerable sources of error which have already been examined.

Rhode Island eggs were hatched at 38·0–40·0° in an incubator, and on various days of incubation the fermentable, and copper-lime precipitable reductions were estimated (*a*) in the alcohol-soluble fraction, (*b*) in the water-soluble fraction of the alcohol precipitate, (*c*) in the water-insoluble fraction of the alcohol precipitate of the whole egg. Glycogen estimations were carried out on separate eggs of the same series. In the more advanced stages of development the embryo was ground up in a mortar containing cold alcohol. All figures are calculated for eggs weighing 50 g. without their shell at the start of incubation.

Alcohol-soluble fraction. The free sugar declines during the first half of incubation but rises again slightly in the last days (Table IX). The minimum was reached somewhat earlier in the series incubated during May–July than in those observed during January–March. In some cases, during the first days of development, considerable differences were met with, even within the same series; later on, the figures agreed fairly well. The rise observed during the last days, though not very great, is considered important, because it occurred in all series followed up to that stage (IV, V, VI, VII, X).

Table IX. *The fermentable reduction of the alcohol extract* (*free sugar*).

Days Series	0	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Season
I	100	87	—	—	34	—	—	28	—	—	—	—	—	—	—	—	—	—	—	Jan.–Mar.
	103	100	—	—	45	—	—	32	—	—	—	—	—	—	—	—	—	—	—	
II	110	71	—	—	50	—	—	30	—	—	11	—	—	—	—	—	—	—	—	
	106	—	—	—	56	—	—	35	—	—	13	—	—	—	—	—	—	—	—	
III	108	97	—	—	74	—	—	31	—	—	22	—	—	13	—	—	—	—	—	
	114	93	—	—	44	—	—	32	—	—	17	—	—	11	—	—	—	—	—	
IV	100	90	—	—	45	—	—	25	—	17	—	—	12	—	—	22	—	—	17	
	108	—	—	—	52	—	—	—	—	19	—	—	16	—	—	20	—	—	23	
V	110	104	—	—	—	27	—	—	—	13	—	—	—	11	—	—	—	15	—	
	115	96	—	—	—	30	—	—	—	18	—	—	—	9	—	—	—	18	—	
VI	—	—	—	—	—	—	—	—	—	8	—	—	—	13	—	—	—	17	—	May–July
	—	—	—	—	—	—	—	—	—	9	—	—	—	14	—	—	—	18	—	
VII	106	98	81	—	—	—	21	—	—	—	—	—	—	—	6	—	—	16	—	
	98	—	81	—	—	—	34	—	—	—	—	—	—	—	7	—	—	15	—	
VIII	—	—	—	—	—	—	—	—	6	—	—	16	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	13	—	—	12	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	—	15	—	—	—	—	—	—	—	—	—	—	
IX	—	—	—	—	—	21	—	—	9	—	—	—	—	—	—	—	—	—	—	
	—	—	—	—	—	36	—	—	11	—	—	—	—	—	—	—	—	—	—	
X	—	—	—	—	51	23	—	10	8	—	—	—	—	—	—	16	—	—	—	
	—	—	—	—	33	22	—	10	8	—	—	—	—	—	—	17	—	—	—	
	—	—	—	—	—	—	—	11	—	—	—	—	—	—	—	—	—	—	—	
Av.	109	92	81	—	48	26	27	24	11	14	15	14	14	12	6·5	19	—	16·5	20	

As shown previously (Part I) the starting level of free sugar is considerably lower than that found by other workers. The divergence of results between the present experiments and those published so far becomes even greater during incubation. Idzumi [1924] states that free sugar falls from 184 to 26 mg. on the 10th day and rises again steadily to 134 mg. on the 20th. Sakuragi [1917] found 200 mg. of free sugar in the fresh egg; this decreased to 19 mg. on the 10th day, subsequently rising to 75–107 mg. on the 13th and 120–162 mg. on the 20th day. Needham's [1927; 1931] smoothed curve starts at 200 mg., reaches its minimum on the 10th day with 33 mg. and rises steadily to 137 mg. on the 20th day. Sagara's [1930] figures show no rise in the second half of incubation; they decline steadily from 173 to 40 mg. on the 10th and to 21 mg. on the 20th day. The fact that Needham uses for his smoothed curves and calculations the data of Idzumi and of Sakuragi for free sugar naturally diminishes their value.

Water-soluble fraction of the alcohol precipitate. Table X shows that the seasonal difference previously noted persists throughout development. Figures were considerably higher in eggs analysed from January to March than in those from May to July. In the same series the values agree fairly well, and they decline in the first half of incubation and rise again in the second. The water-soluble fraction of the alcohol precipitate can be subdivided into three parts

Table X. *The water-soluble fraction of the alcohol precipitate.*

Reduction precipitable with copper-lime.

Days Series	0	3	4	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Season
I	84	77	—	97	—	—	86	—	—	85	—	—	—	—	—	—	—	—	Jan.–Mar.
	89	81	—	76	—	—	82	—	—	83	—	—	—	—	—	—	—	—	
II	81	80	—	76	—	—	71	—	—	67	—	—	—	—	—	—	—	—	
	79	83	—	83	—	—	67	—	—	58	—	—	—	—	—	—	—	—	
III	86	80	—	68	—	—	82	—	—	77	—	—	91	—	—	—	—	—	
	78	91	—	73	—	—	70	—	—	65	—	—	75	—	—	—	—	—	
IV	76	88	—	69	—	—	62	—	67	—	—	98	—	—	113	—	—	98	
	82	89	—	65	—	—	60	—	79	—	—	110	—	—	104	—	—	106	
V	98	101	—	—	61	—	—	—	88	—	—	—	73	—	—	—	—	—	
	88	92	—	—	62	—	—	—	87	—	—	—	83	—	—	—	—	—	
VI	—	—	—	—	—	—	—	—	—	—	—	—	82	—	—	—	95	—	
	—	—	—	—	—	—	—	—	—	—	—	—	92	—	—	—	100	—	
VII	—	—	—	—	—	—	—	—	98	—	—	—	80	—	—	—	94	—	
	—	—	—	—	—	—	—	—	70	—	—	—	77	—	—	—	78	—	
VIII	95	—	99	—	—	72	—	—	—	—	—	—	—	—	—	—	—	—	
	90	—	93	—	—	64	—	—	—	—	—	—	—	—	—	—	—	—	
I*a*	47	—	—	—	—	—	—	—	—	—	—	—	—	56	—	66	69	—	May–July
	57	—	—	—	—	—	—	—	—	—	—	—	—	61	—	63	66	—	
II*a*	58	—	—	—	—	—	—	41	—	—	53	—	—	—	—	—	—	—	
	50	—	—	—	—	—	—	49	—	—	35	—	—	—	—	—	—	—	
	—	—	—	—	—	—	—	58	—	—	35	—	—	—	—	—	—	—	
III*a*	48	—	—	—	51	—	—	44	—	—	—	—	—	—	—	—	—	—	
	54	—	—	—	46	—	—	46	—	—	—	—	—	—	—	—	—	—	
	—	—	—	—	50	—	—	—	—	—	—	—	—	—	—	—	—	—	
IV*a*	50	—	56	47	—	—	—	45	37	—	—	—	—	—	54	—	—	—	
	56	—	56	60	—	—	—	49	54	—	—	—	—	—	55	—	—	—	
	49	—	73	51	—	—	—	46	—	—	—	—	—	—	—	—	—	—	
Average Jan.–Mar.	88	86	—	77	65		72	—	77		—	—	82	—	—	—	92	—	
Average May–July	52	—	—	51		—	—	47	—	—	—	—	—	56		66		—	

(Part I) (*a*) not precipitable by phosphotungstic acid, (*b*) precipitable with phosphotungstic, but not with trichloroacetic acid, (*c*) precipitable with trichloroacetic acid. Fig. 1 shows the relative amounts of fractions *a*–*c* present during incubation in % of the water-soluble fraction. The initial fall in the latter is, as Fig. 1 shows, not due to fraction (*b*), consisting mainly of ovomucoid glucose, but to fractions (*a*) and (*c*). Fraction (*a*) contains probably no carbohydrate. The rise observed during the second half of incubation is mainly due to fractions (*a*) and (*b*), as (*c*) falls off again in the last days to reach its lowest level. Leaving fraction (*a*) out of consideration as probably non-carbohydrate, the observed increase could be accounted for by the accumulation of glycogen, but direct estimations show that only a small fraction of the egg's glycogen is present in the water-soluble fraction. Fermentation shows that the observed increase is only partly fermentable; so the conclusion seems warranted, that a part of the increase is due to the formation of a substance similar to that contained originally in the water-soluble fraction.

Water-insoluble fraction of the alcohol precipitate. Contrary to the case with other fractions, figures obtained here vary widely, so that some difficulty is encountered in calculating averages. Nevertheless, we can state with confidence

Table XI. *The water-insoluble fraction of the alcohol precipitate.*

Fermentable reduction.

Days Series	0	4	6	7	8	9	10	11	13	14	16	17	18	19
I	—	—	—	—	—	85	—	—	—	—	—	—	—	44
	—	—	—	—	—	82	—	—	—	—	—	—	—	43
II	94	—	—	—	—	—	—	82	—	55	—	—	—	47
	101	—	—	—	—	—	—	86	—	58	—	—	—	41
III	83	56	—	—	54	—	—	—	—	—	—	45	—	—
	79	54	—	—	57	—	—	—	—	—	—	49	—	—
IV	78	—	—	—	—	—	—	—	—	—	80	—	—	—
	80	—	—	—	—	—	—	—	—	—	79	—	—	—
V	83	—	—	—	—	—	45	—	70	—	—	—	—	—
	78	—	—	—	—	—	45	—	62	—	—	—	—	—
		—	—	—	—	—	48	—	60	—	—	—	—	—
VI	—	—	—	51	—	—	39	—	—	—	—	—	—	—
	—	—	—	59	—	—	35	—	—	—	—	—	—	—
	—	—	—	64	—	—	—	—	—	—	—	—	—	—
VII	—	65	47	—	—	—	60	—	—	—	50	—	—	—
	—	61	41	—	—	—	66	—	—	—	56	—	—	—
	—	66	40	—	—	—	—	—	—	—	—	—	—	—
VIII	—	—	—	—	—	41	—	—	—	—	—	—	50	—
	—	—	—	—	—	43	—	—	—	—	—	—	45	—
	—	—	—	—	—	33	—	—	—	—	—	—	—	—
IX	—	—	—	[8*	—	—	62	—	—	—	—	—	—	—
	—	—	—	18]	—	—	63	—	—	—	—	—	—	—
	—	—	—	—	—	—	67	—	—	—	—	—	—	—
Average	84	60	50		57		58		60		58		45	

* Not included in averages.

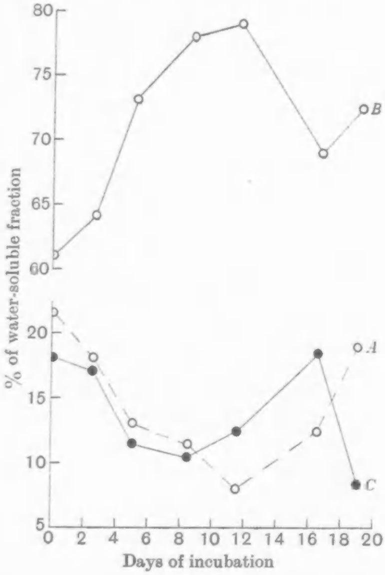

Fig. 1.

that this fraction has diminished by the end of incubation, though in many cases a considerable fluctuation may occur during the process. The variability of these results could be explained by a great individual variability or by a very large experimental error. The fact that results obtained in the same series of eggs agree closely rather suggests that such error does not occur, and that this variability is based on some metabolic process. It is obvious that, for certain purposes, only results obtained in the same series can be compared. The significance of these increases (series V, VII, IX of Table XI) will be considered later.

The carbohydrate of this fraction changes during development. At the start of incubation only 4–5 mg. of the fermentable reduction (4–6 %) is due to glycogen; at the end the glycogen represents 80–100 % of the fermentable reduction.

Table XII. *Glycogen.*

(January to March.)

Days	0	4	6	7	8	9	10	11	14	15	16	17	19	20
Series														
I	9·6	—	—	—	—	17	—	24	30	—	—	42	—	60
	9·0	—	—	—	—	11	—	21	36	—	—	37	—	68
	10·0	—	—	—	—	—	—	—	—	—	—	—	—	—
II	8·2	—	9	10	—	—	19	—	31	—	—	—	—	—
	8·4	—	15	8	—	—	19	—	25	—	—	—	—	—
	—	—	—	—	—	—	15	—	25	—	—	—	—	—
III	10·6	—	—	—	—	—	—	33	—	41	—	—	56	—
	11·9	—	—	—	—	—	—	20	—	42	—	—	58	—
IV	7·2	6·8	—	—	9	—	—	—	—	—	44	—	—	—
	7·6	10·2	—	—	10	—	—	—	—	—	35	—	—	—
	8·0	—	—	—	—	—	—	—	—	—	—	—	—	—
Average	9·0	8·5	10·5		12		18	24	29	40			57	64

Table XII shows the steady accumulation of glycogen throughout incubation. The figures given are considerably higher than those of other workers [Idzumi, 1924; Sakuragi, 1917; Murray, 1926]. A seasonal difference could possibly account for this, as all estimations here considered were done from January to March, and in two experiments done in July, lower figures were found on the 19th day (30–40 mg.).

Table XIII. *Total carbohydrate.*

Without fraction (*a*) of water-soluble fraction.

Days	0	3	6–7	8–9	10	11–12	13–14	15	17–18	19–20
Total carbohydrate	261	222	161	146	140	143	144	142	146	137

Table XIII demonstrates that the total carbohydrate content of the egg is practically constant from the 8–9th day to the end of incubation and shows no trace of the accumulation described by Needham [1927], who found, after an initial value of 335 mg., on the 6th day 152, on the 7th 142, on the 8th 145 mg. followed by a subsequent rise to 234 mg. on the 11th day. After the 11th day his total carbohydrate fell to 177 mg. on the 20th. In another of Needham's series (using the Wood-Ost method), the fall is smaller; the respective figures are 253 mg. on the 10th and 235 mg. on the 20th day. Sakuragi's [1917] figures show no rise between the 7th and 11th days but a steady decline from 426 mg. on the 6th to 348 mg. on the 20th day. Sagara's [1930] figures fall steadily from 398 to 94 mg. during incubation.

Discussion.

The source of glycogen. It is evident from the figures included in Table IX that free sugar is not the source of the glycogen. Free sugar reaches the lowest level on the 10th day, when only about 18 mg. of glycogen are present. The major part of the glycogen is formed between the 10th and 20th days (Table XII), in which period free sugar remains unchanged at first and even increases a little during the last days. Neither is glycogen formed to any considerable extent from the reducing substances contained in the water-soluble fraction of the alcohol precipitate, as (1), though the major part of the glycogen is found in the water-insoluble fraction, the reducing power of the water-soluble fraction does not diminish, (2) any glycogen present in this fraction is accounted for by the increase in reduction during the second half of incubation, (3) a formation of glycogen would change the ratio of fermentable reduction and the reduction precipitable with copper-lime; the change actually observed is very limited, (4) in many cases the total fermentable reduction of this fraction does not account for all the glycogen formed. There remains as a source of glycogen the water-insoluble fraction of the alcohol precipitate. The fermentable reduction of this fraction suffices to account for all the glycogen formed, and direct estimations show that in the later stages of development about 80–90 % of the glycogen is contained in this fraction.

It is impossible to decide whether substances which have no reducing power even after hydrolysis do not play some part in the formation of glycogen. But, as a preformed, reducing and fermentable substance accounts for all the glycogen, the supposition of direct glycogen formation from other sources does not seem necessary.

Gluconeogenesis. The evidence brought forward by Needham [1927], according to which about 90 mg. of sugar are formed from fat between the 7th and 11th days, could not be confirmed by the present experiments. The average figures for total carbohydrate (Table XIII) are practically constant from the 8th to 9th days and so furnish no direct evidence for a gluconeogenetic process; yet they would mean that, without the assumption of gluconeogenesis during the second half of incubation, no carbohydrate is consumed at all, a supposition scarcely tenable. Attention was previously directed to the change taking place in the fermentable reduction of the water-insoluble fraction of the alcohol precipitate (Table XI). In some series (V, VII, IX) a considerable increase in this fraction was observed, which was not accompanied by an equivalent decrease in any other fraction. Table XI shows that, within one and the same series of eggs, all the changes were fairly uniform. The fact that this fraction is the main source of glycogen emphasises the importance of these changes. These data seem to afford some evidence that gluconeogenesis is going on during incubation, though on the whole the total carbohydrate remains unchanged, implying that the breaking down and the new formation of carbohydrate are usually in equilibrium.

Summary.

The fermentable and copper-lime-precipitable reductions were estimated in the hen's egg during development:

(*a*) In the alcohol-soluble fraction.

(*b*) In the water-soluble fraction of the alcohol precipitate.

(*c*) In the water-insoluble fraction of the alcohol precipitate.

(*d*) Glycogen estimations were carried out on separate eggs of the same series.

The following conclusions are arrived at.

1. Free sugar reaches its lowest level on the 10th day and rises again slightly during the last days of incubation.
2. Reduction in the water-soluble fraction of the alcohol fraction of the alcohol precipitate decreases in the first and increases in the second half of incubation. This increase is probably not due wholly to carbohydrate and is partly accounted for by the accumulation of glycogen.
3. The reducing power of the water-insoluble fraction of the alcohol precipitate decreases on the whole from the start to the end of incubation, though considerable increases may occur in between. This fraction is the principal source of the glycogen formed during incubation.
4. Total carbohydrate diminishes till the 9th day and remains constant later.
5. Though no direct evidence could be obtained from the average figures, the changes observed in the water-insoluble fraction of the alcohol precipitate combined with other considerations warrant the supposition of gluconeogenesis.

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CVIII. A NEW METHOD FOR THE DETERMINATION OF CELLULOSE, BASED UPON OBSERVATIONS ON THE REMOVAL OF LIGNIN AND OTHER ENCRUSTING MATERIALS.

By ARTHUR GEOFFREY NORMAN
and SAMUEL HARRY JENKINS.

From the Fermentation Department, Rothamsted Experimental Station, Harpenden, Herts.

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In the analysis of plant materials and woods, one of the chief constituents to be determined is cellulose, which is important since it forms the structural framework of the tissue. The determination of cellulose, however, is for various reasons not always satisfactory. The methods of determination of cellulose in general use do not all give the same product; consequently the results obtained are not always directly comparable. The method selected may be dictated by the purpose for which the cellulose is required. As it occurs naturally, cellulose is always found in the closest association with other constituents of the cell-wall, and these have, by various treatments, to be removed. Originally it was held that a number of distinct compound celluloses existed, *e.g.* lignocelluloses, pectocelluloses, *etc.* [Cross and Bevan, 1895], but the more recent view, which is generally accepted, is that all natural celluloses contain a certain fraction which is identical chemically. This fraction, typified by pure cotton cellulose, will from different sources give identical X-ray diagrams, comparable yields of cellobiose octa-acetate, and solely glucose on hydrolysis. It may therefore be regarded as "true" cellulose or "pure" cellulose in the rigid chemical sense. But cellulose as usually isolated does not yield only glucose on hydrolysis. In the case of hardwoods, cereal straws and certain fibre plants, xylose also is obtained, while in the case of softwoods mannose may be found in addition. These sugars are derived from polysaccharide material very intimately associated with the "pure" cellulose itself, and held either by adsorption or secondary valency forces. The association is so close that these polysaccharides may only be completely removed intact by the most violent treatment with alkali. Mild acid hydrolysis on the other hand removes them rapidly. These associated polysaccharides, which possess the general properties usually described for the hemicelluloses, have been termed by Hawley and Norman [1932] "cellulosans" and will be referred to as such in this paper. Development studies on the barley plant [Norman, 1933] show that the cellulosans are laid down with the "true" cellulose from the earliest stages and are not produced as the result of changes during lignification or senescence. The inference from this and other work is that the cellulosans must be regarded as forming a normal integral part of the cellulosic fabric of the plant, *i.e.* natural celluloses consist of "true" cellulose *plus* associated cellulosan. These two substances together will be taken in this paper to be covered by the word "cellulose."

As ordinarily found, natural cellulose is associated or encrusted with lignin and hemicelluloses of a polyuronide nature. Recent work on the molecular anatomy of "true" cellulose, as revealed by X-ray studies, makes it unlikely that primary valency linkages are involved in the association of lignin and cellulose, and physical theories of adsorption are usually proposed. Methods of cellulose determination therefore involve the removal of this lignin and encrusting hemicelluloses. The requirements of an ideal method are that lignin and polyuronide hemicelluloses should be rapidly and completely removed without affecting the integrity of the natural cellulose. It is in this last respect that a number of existing methods fail, inasmuch as they involve some treatment which attacks the less resistant cellulosan fraction.

The effect of pre-treatment on the yield of cellulose.

The first and by far the most important method is the chlorination process of Cross and Bevan, in which, by exposure to gaseous chlorine, a lignin-halogen derivative is formed and subsequently dissolved out together with the hemicelluloses by boiling with neutral sulphite. Alternate chlorination and boiling with sulphite have to be continued till, on addition of the sulphite, there is no red colour reaction indicative of lignin. In the case of many woods and fibres some three or four brief chlorinations are sufficient, but with the limited number of woods used in this work the number was considerably higher. Cross and Bevan [1895] originally proposed an alkaline pre-treatment of the material before chlorination, and boiled for 20 minutes with 1 % NaOH. Wood chemists in their official methods have, however, dispensed with this pre-treatment on the ground that the yield of cellulose is lowered thereby, as claimed by Renker [1910]. In the case of certain non-timber materials, as, for instance, cereal straws, chlorination without pre-treatment is an extremely tedious operation, in that as many as 9 or 10 exposures to chlorine and subsequent extractions with sulphite may be required, during which the cellulose becomes gelatinous and difficult to handle. In these cases an alkaline boiling is of great advantage, since the number of chlorinations then necessary is reduced to 2 or 3. Dorée [1933], discussing this point, advocates boiling for 5 minutes with 1 % NaOH on the grounds that it enormously facilitates subsequent delignification. It has been claimed that in the case of certain woods a lignin-free cellulose product is not attainable without prior alkaline treatment [*cf.* Strong, 1928]. Norman [1929], in work on the decomposition of cereal straws, employed the original Cross and Bevan procedure of boiling for 20 minutes with 1 % NaOH. There is little doubt, however, that advantageous as is this pre-treatment in expediting the removal of lignin, it is objectionable in that the cellulosan fraction may be partly attacked and suffer loss. Hawley and Norman [1932] drew attention to this on theoretical grounds and some experimental evidence on the point is given in Table I. Samples of three cereal straws were extracted for 20 minutes with boiling 1 % NaOH, filtered and washed thoroughly with hot water and a little dilute acetic acid. After drying and grinding, cellulose was determined by the Cross and Bevan method without alkaline pre-treatment. Total furfuraldehyde, lignin and furfuraldehyde due to cellulosan were also determined.

The effect of the alkaline pre-treatment on the cellulose yield is seen, as expected, to be a depressant one, the figures after treatment being less by the amount of cellulosan extracted. A measure of this is obtained from the figures for furfuraldehyde from the cellulose products, inasmuch as the cellulosan in the case of these three straws is a xylan. That subsequent delignification is

Table I. *Effect on cellulose determination of pre-treatment of straws with alkali. (Each straw boiled for 20 minutes with 1 % NaOH.)*

All results expressed on the basis of 100 g. original dry straw.

Straw	Residue after extraction	Cellulose after extraction*	Furfurald. from cellulose after extraction	Cellulose on original straw	Furfurald. from cellulose on original straw	Apparent lignin on original straw	Apparent lignin after extraction	Total furfurald. on original straw	Total furfurald. after extraction	Non-cellulosic furfurald. on original straw	Non-cellulosic furfurald. after extraction
Oats	52·00	48·7	7·09	53·4	9·30	19·99	5·14 (74·30)	15·86	7·99 (49·60)	6·53	0·90 (86·20)
Wheat	62·52	53·7	9·55	55·2	9·96	20·93	6·02 (71·20)	16·64	10·58 (36·40)	6·68	1·03 (84·60)
Rye	66·31	57·2	10·18	60·2	11·44	21·56	6·97 (67·70)	14·80	12·05 (18·60)	3·36	1·87 (44·20)

* Cellulose determined by Cross and Bevan method without pre-treatment.

Figures in brackets represent percentage removed by pre-treatment with alkali.

facilitated is not surprising since the alkali treatment itself removes approximately 70 % of the total lignin of each straw. In addition a considerable proportion of the non-cellulosic furfuraldehyde-yielding constituents—the polyuronides—is removed by the pre-treatment.

Because of the difficulties mentioned above in determining cellulose in cereal straws by the Cross and Bevan procedure, Jenkins [1930] proposed a method involving the use of hypochlorite. Before submitting the straw to this reagent, however, it is boiled alternately for 5 minutes each with 1 % NaOH and 1 % HCl, these treatments being repeated twice. Jenkins [1930] quotes figures to show that the method gives results definitely lower than does Cross and Bevan's, but that this difference is due to loss of cellulosan rather than attack on "true" cellulose, which is identical in both cases. Unfortunately the figures were compared against the chlorination method with an alkaline pre-treatment. Had this been omitted, it would have been seen that the loss of cellulosan was great, and no doubt due to the hydrolysing effect of the acid boiling. To demonstrate in detail the losses occurring in each stage of the procedure advocated by Jenkins [1930] a large sample of straw was put through the process and analyses were made at each stage. By the Jenkins method the straw gave a "cellulose" figure of 39·56 %. The results are given in Table II.

Table II. *Analysis of effects of pre-treatments in Jenkins's cellulose method, using oat straw.*

All results expressed on basis of 100 g. original straw.

Treatment	Residue	Cellulose *	Furfurald. on cellulose	Total furfurald.	Non-cellulosic furfurald.	Apparent lignin †
None	—	53·5	9·2	15·9	6·7	19·9
Extracted twice with 1 % NaOH (5 mins.)	49·9	42·2 (21·2)	6·2 (33·2)	8·3	2·1 (68·7)	4·4 (77·9)
Extracted twice with 1 % HCl (5 mins.)	62·2	43·3 (19·2)	4·9 (47·2)	8·3	3·4 (49·2)	13·5 (32·2)
Extracted twice with alkali and acid (5 mins. each)	41·6	37·8 (29·3)	4·4 (51·9)	5·1	0·7 (89·5)	4·0 (79·9)

* By method described later in this paper.

† By 72 % sulphuric acid method.

Figures in brackets represent the percentage loss due to the treatment.

Although the individual treatments are brief the hydrolysing and extracting effects produced by them are very considerable. Alkali extracts more than does acid, mainly by reason of the extra lignin which it removes. The total furfuraldehyde yields of both acid- and alkali-extracted straw are identical but made up in rather a different way. The acid extraction apparently removes by hydrolysis over 45 % of the cellulosan, and alkali by solution less than 35 %, while on the other hand the effects of the two treatments on the non-cellulosic furfuraldehyde-yielding constituents are in the opposite direction. The apparent loss of lignin on brief treatment with acid was quite unexpected and will be referred to later. The combined effect of alkali and acid is a very drastic extraction of the straw, which loses nearly 70 % of its total furfuraldehyde-yielding groups and 80 % of its apparent lignin. The most serious result from the point of view of the subsequent determination of cellulose is that, by calculation from the furfuraldehyde in the cellulose, nearly 50 % of the cellulosan is removed. Pure cellulose, as typified by a high grade cotton, was shown by Jenkins [1930] to be unaffected by this treatment. The result of the subsequent three hypochlorite treatments is evidently very slight in comparison with that of the pre-treatments, for in this case the residue after the latter was 41·6 %, which was reduced to 39·56 % by the hypochlorite. Because of this slight effect of hypochlorite it was thought to be worth while to determine the lignin content of celluloses prepared by the Jenkins procedure. Some figures for straws are given in Table III, together with those for the Cross and Bevan product from the same material.

Table III. *Comparison of cellulose products obtained by Jenkins's method with those given by the Cross and Bevan procedure without pre-treatment.*

Straw	Jenkins cellulose in 100 g. straw	Furfurald. in 100 g. Jenkins cellulose	Apparent lignin in 100 g. Jenkins cellulose	C. and B. cellulose in 100 g. straw	Furfurald. in 100 g. C. and B. cellulose	Apparent lignin in 100 g. C. and B. cellulose
Oats A	41·9	9·1	4·8	53·5	16·8	3·0
Wheat A	47·5	9·9	7·8	55·2	17·7	3·1
Wheat B	45·1	10·4	4·6	56·7	18·3	2·9
Barley	35·2	8·9	3·9	47·7	—	3·3
Rye	51·9	10·9	6·6	60·2	19·0	2·4

Apart from the serious losses of cellulosan, to which attention has been directed above, it will be seen that the lignin figures given by celluloses prepared by the Jenkins procedure are hardly satisfactory. Even those obtained on the Cross and Bevan products are not as low as expected, an observation which will be dealt with in more detail below.

Therefore, although the method proposed by Jenkins [1930] may be suitable as a rapid and arbitrary procedure for determining a certain fraction of straws, a fraction not dissimilar from that given by the old-established crude fibre determinations, it cannot be regarded as providing a real indication of the cellulosic fabric of the material. It is faulty in two respects; firstly that the cellulosan fraction is very heavily attacked, and secondly that the product is not completely delignified.

The rôle of sulphite in the determination of cellulose by a chlorination procedure.

The chlorination methods of cellulose determination, as previously stated, depend on alternate exposures to gaseous chlorine and extraction with boiling sodium sulphite in which the lignin-chlorine complex is soluble. At the same time the hemicelluloses are removed by the boiling sulphite. It seemed to be of interest therefore to determine to what extent the chlorination itself is important. Samples of oat straw were given 1, 3 and 6 treatments with boiling sulphite for 20 minutes and the residues analysed for lignin and total furfuraldehyde. At the same time three other samples were given 1, 3 and 6 gaseous chlorinations for 10 minutes, each followed by boiling with sulphite for 20 minutes. The results are given in Table IV. Six neutral sulphite treatments alone remove 74 % of the lignin but only 22 % of the total furfuraldehyde-yielding constituents. The effect of the chlorination is greatly to expedite the removal of lignin, since at the end of the third chlorination as much as 83 % has been extracted. The chlorine however also has a marked effect on the removal of hemicelluloses, as indicated by the furfuraldehyde figures. Three chlorinations lower the furfuraldehyde yield by 40 % while three sulphite treatments alone only remove 15 %. This effect of chlorine in assisting in the removal of the hemicelluloses was unexpected and has not been commented upon before. It seems that the chlorination is at least as important in this respect as in removing lignin. Its mode of action is not understood at present, but will be the subject of further investigation. Neutral sulphite without chlorination has been proposed as a method of delignification of pulps but has not been found satisfactory, since high pressures and concentrations are necessary to obtain a satisfactory product [Rawling and Staidl, 1925].

Table IV. *Effect on oat straw of sulphite boils with and without chlorination.*

Treatment	Residue	Furfurald. on residue	Apparent lignin on residue	Furfurald. on original straw basis	Apparent lignin on original straw basis
1 chlorination* 1 sulphite boiling	73·4	19·2	12·2	14·1 (11·2)	8·9 (55·2)
1 sulphite boiling	75·6	20·4	14·8	15·4 (2·8)	11·2 (43·8)
3 chlorinations 3 sulphite boilings	58·2	16·4	5·7	9·5 (39·9)	3·3 (83·4)
3 sulphite boilings	68·4	19·6	12·5	13·4 (15·5)	8·5 (57·2)
6 chlorinations 6 sulphite boilings†	52·2	17·2	4·4	9·0 (43·5)	2·3 (88·5)
6 sulphite boilings	62·7	19·7	8·5	12·4 (22·2)	5·2 (74·0)

* Chlorination by gaseous Cl—10 mins., 3 % sulphite boiling—20 mins.
† Still gave sulphite reaction for lignin.
Figures in brackets give percentage loss by treatment.

Proposed new method for the determination of cellulose.

In view of the many chlorinations to which it is necessary to subject a cereal straw in order to obtain satisfactory delignification without pre-treatment, an attempt was made to devise a method more suited to this type of material. Jenkins [1930] observed that lignin could be removed in part by the use of sodium hypochlorite. It was found, however, that the delignifying effect could be enhanced by following the hypochlorite treatment by boiling with sodium sulphite, just as is done in the Cross and Bevan procedure. No colour reaction with sulphite, such as is given after treatment with chlorine gas, is observed.

The mode of action of the hypochlorite is not clear, and it can only be supposed that a soluble additive compound with the whole lignin molecule is obtained. After preliminary boiling with water, about 2 g. straw were allowed to stand for 15 minutes in 100 cc. water to which had been added 5 cc. hypochlorite containing approximately 15 % available chlorine[1]. The straw was then filtered off on cloth and boiled for 20 minutes with 100 cc. 3 % sodium sulphite. This process was carried out one, two, three, four and six times on a sample of oat straw, with the results given below. Though the products after 4 and 6 hypochlorite treatments were excellent in appearance and retained the original structure they did not compare favourably in lignin content with those obtained by the Cross and Bevan procedure. Moreover, when exposed to gaseous chlorine, the product after 6 neutral hypochlorite treatments gave an intense purple reaction for lignin on addition of sulphite. Further, on subjecting different straws to 4 hypochlorite treatments, the effectiveness of the treatment was found to vary considerably, as will be seen in Table VI.

Table V. *Effect on oat straw of neutral hypochlorite treatments followed by sulphite boiling.*

Procedure	Cellulosic product	Apparent lignin % on cellulose
1 hypochlorite treatment	69·92	14·8
2 hypochlorite treatments	63·24	9·2
3 ,,	54·80	9·5
4 ,,	54·04	6·9
6 ,,	53·66	4·7
C. and B. 9 chlorinations without pre-treatment	53·99	3·1

Table VI. *Comparison of products of four neutral hypochlorite treatments with those obtained by the Cross and Bevan method.*

	Hypochlorite method		Cross and Bevan method	
Straw	Cellulose product	Lignin on product	Cellulose product	Lignin on product
Oats A	54·0	6·9	53·4	3·1
Oats B	53·1	4·3	53·5	3·0
Wheat A	56·2	5·2	55·2	3·1
Wheat B	59·6	7·4	56·7	2·9
Barley	48·3	6·7	47·7	3·3
Rye	65·6	9·1	60·2	2·4

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[1] An excellent and inexpensive solution of sodium hypochlorite may be obtained from Messrs Laporte of Luton. It contains hypochlorite equivalent to 15–17 % available chlorine, together with about 14 % NaCl, 1 % NaOH and 1 % Na_2CO_3. The strength of the hypochlorite may readily be determined by titration with thiosulphate in the presence of KI and H_2SO_4. The available chlorine or chlorine liberated on acidification represents twice the amount actually present as NaOCl, as shown by the equations

$$NaOCl + HCl = NaCl + HOCl. \quad HOCl + HCl = Cl_2 + H_2O.$$

Owing to the presence of NaCl in the solution the same relationship holds on acidification with sulphuric or any other strong acid.

$$NaOCl + 2H_2SO_4 + NaCl = 2NaHSO_4 + H_2O + Cl_2.$$

The equations given above have been verified experimentally.

In the absence of any easy and reliable test for lignin, clearly a method which involved a different number of treatments with different materials could not be very satisfactory. Accordingly it was decided to introduce a treatment with free chlorine so that the sulphite colour reaction for lignin might be employed and in the hope that the lignin might more completely be removed. The free chlorine was obtained by adding to hypochlorite an amount of sulphuric acid somewhat in excess of the equivalent so that hypochlorous acid should not be present to any extent. When cold sulphite is added to the straw from this acid hypochlorite treatment, if lignin is still present, a red purple colour develops just as if exposed to chlorine gas. In straws which have previously undergone a treatment with neutral hypochlorite and sulphite, the colour is more definitely purple than the red-brown colour usually obtained after chlorine gas. This suggests that the neutral hypochlorite removes some material or some portion of the lignin masking the purplish tint. Many experiments were carried out to determine the most satisfactory number combination of neutral[1] and acid chlorination treatments. To be practical it was considered that the number ought not in normal circumstances to exceed five, and accordingly the estimations were stopped at that point whether or not a sulphite reaction for lignin was still given. The results of some of the combinations of neutral and acid hypochlorite treatments are shown in Table VII.

Table VII. *Comparison of results of various combinations of neutral and acid hypochlorite treatment of oat straw.*

Treatment*	Cellulose on straw	Apparent lignin on cellulose	Lignin reaction at end
C. and B. 9 chlorinations	53·4	3·1	None
A.A.A.A.A. (2 mins. A.)	55·6	3·6	None
N.N.A.A.A. (2 mins. A.)	54·1	3·7	None
A.N.A.N.A. (2 mins. A.)	55·5	3·9	Slight
N.N.N.A.A. (2 mins. A.)	56·1	3·5	Definite
N.N.A.N.A. (2 mins. A.)	54·7	3·8	Trace
A.N.N.A.A. (2 mins. A.)	56·0	3·7	Strong
A.A.A.A.A. (15 mins. A.)	54·8	3·4	Slight
N.N.A.A.A. (15 mins. A.)	53·6	3·1	None
N.N.A.A.A. (5 mins. A.)	55·0	3·3	Slight
N.N.A.A.A.G. (5 mins. A.)	53·1	3·0	None
N.′N.′A.A.A. (10 mins. A.)	53·5	3·5	None

* N. 5 cc. NaOCl (15 % avail. Cl.) to 100 cc. water for 5 minutes, filtered and followed by sulphite boiling for 10 minutes.
A. 5 cc. NaOCl (3 % avail. Cl.) to 100 cc. water + 2 cc. 20 % H_2SO_4, filtered and followed by sulphite boiling for 10 minutes.
G. Exposure to chlorine gas for 5 minutes.
N.′ As N. but for 10 minutes.

Chlorination with acid hypochlorite under similar conditions was found to be harmless to pure cellulose. Finely ground filter-paper given three such treatments for 5 minutes followed each time by boiling for 10 minutes with sulphite suffered a loss of only 2·7 %. Controls which were given three sulphite boilings without chlorinations lost precisely the same amount.

Arising from Table VII, the following method was adopted and found to be applicable not only to straws but also to woods. The material should be in a uniform and fine state of division; in the case of woods 60–80 mesh appears to be most suitable, while in the case of straws 40–60 seems preferable. Woods must be given an alcohol-benzene extraction but this is unnecessary with cereal straws. About 2 g. material are brought to the boil in 100 cc. 3 % sulphite and filtered off on fine smooth poplin stretched over a Büchner funnel about 6 cm. in diameter and closely fastened either in a ring or with tight rubber bands. This preliminary sulphite boiling is advantageous not only in ensuring thorough wetting of the material but also in that a certain amount of lignin is removed, as shown in Table IV. Furthermore straws boiled with sulphite often filter much more readily than if treated only with water. The filtered material is then removed to a beaker by means of a spatula and jet from a wash-bottle. After making up the filtered straw to 100 cc., 5 cc. sodium hypochlorite (approx. 15 % available chlorine) are added and allowed to stand for 10 minutes. The material is filtered off, again transferred to a beaker and made up to 50 cc. with water; 50 cc. 6 % sodium sulphite are added, and the whole boiled for 20 minutes. It is usually convenient to bring to the boil over a flame and then to stand the beaker in a boiling water-bath, as bumping is frequently serious in the later stages. The treatment with hypochlorite is repeated a second time and followed by a sulphite boiling as before. The material, which by this time is much lighter in colour, is again suspended in 100 cc. water, and 5 cc. dilute hypochlorite (3 % available chlorine) are added, together with 2 cc. 20 % sulphuric acid. Chlorine is evolved and the material frequently turns yellow, just as it does in the Cross and Bevan process with gaseous chlorine. After standing for 10 minutes out of direct sunlight it is filtered, made up to 50 cc. and sulphite added as before. An intense purple coloration is given indicating the presence of lignin. This colour disappears on heating and the solution then becomes brown. The treatments with acid hypochlorite are continued as long as a positive reaction for lignin is given on addition of sulphite. In the case of some straws two acid treatments are sufficient. The process should not be continued longer than is necessary as indicated by this reaction. Sulphite is removed by suspension in 200 cc. or so of hot water, and after thorough washing the cellulose product is transferred to a Gooch crucible containing a small circle of cloth. It is dried to constant weight at 100°.

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[1] The treatments are spoken of as neutral and acid respectively for the sake of clarity. The neutral treatments are in fact faintly alkaline by reason of the free alkali in the sodium hypochlorite, necessary for stability.

Chlorination under these conditions is without serious effect on pure cellulose. Samples of Whatman No. 1 filter-paper, finely ground, were given in duplicate the treatment described above but prolonged to the extent of 5, 7 and 10 acid chlorinations to determine the effect, if any, of gross over-chlorination. The recoveries from 1 g. were practically identical and equal to the amount obtained after 6 alternate cold and boiling-water extractions. The latter treatment represents water-soluble material *plus* mechanical losses in handling. These results are given in Table VIII.

Table VIII. *Effect of over-chlorination on pure filter-paper.*

Treatment	Amount recovered from 1 g. cellulose
2 N. 3 A.*	0·958
2 N. 5 A.	0·951
2 N. 7 A.	0·956
2 N. 10 A.	0·952
6 BW. 5 CW.†	0·952
Boiled in water 2 hours	0·991

* Times and concentrations as given in Table VII.

† Boiled for 20 minutes with water, filtered and allowed to stand in cold water for 10 minutes.

Table IX shows results obtained for certain straws and woods by this method, compared with the figures obtained by the Cross and Bevan procedure without pre-treatment. The cellulose figures are the averages of several determinations, while lignin and furfuraldehyde estimations were carried out only in duplicate. From this evidence it is claimed that the method gives results strictly comparable with those of the Cross and Bevan determination without pre-treatment, and that the product represents as closely as is possible the natural cellulosic tissue of the plant freed from encrusting substances. It possesses certain general advantages in that it dispenses with the use of chlorine gas and needs no permanent apparatus. With a sufficiently large water-bath tc take the sulphite extractions after boiling, one worker can carry on as many as 16 determinations and, unless the material is especially resistant, carry them all through to completion in the course of a day. For cereal straws the method is preferable, since the number of chlorinations necessary is lessened, and the product does not become gelatinous or difficult to handle.

Table IX. *Comparison of results obtained on straws and woods by the Cross and Bevan method without pre-treatment and by the new method.*

	Cross and Bevan method				New method			
Material Straws and woods	No. of chlorinations	Cellulose %	Furfurald. on cellulose %	Apparent lignin on cellulose %	No. of chlorinations*	Cellulose %	Furfurald. on cellulose %	Apparent lignin on cellulose %
Oats A	7	53·4	17·4	3·1	2 N. 3 A.	53·5	17·2	3·5
Oats B	7	53·5	16·8	3·0	2 N. 3 A.	52·5	17·0	2·6
Wheat A	7	55·2	17·7	3·1	2 N. 3 A.	55·5	17·6	1·6
Wheat B	6	56·7	18·3	2·9	2 N. 3 A.	56·3	18·2	2·4
Rye	7	60·2	19·0	2·4	2 N. 4 A.	59·8	18·7	2·4
Oak (Danzig)†	10	53·2	18·1	0·3	2 N. 5 A.	53·9	18·2	0·7
Beech†	8	58·8	15·5	1·0	2 N. 5 A.	59·4	15·5	2·1
Scotch Pine†	8	60·2	5·2	0·8	2 N. 5 A.	60·8	5·5	0·2
Sitka Spruce†	6	66·8	3·3	0·2	2 N. 5 A.	65·9	3·2	0·1

* N. Neutral hypochlorite treatment. A. Acid hypochlorite treatment.
† Results expressed on material extracted with alcohol-benzene.

The method is easily applicable to large scale preparations of natural celluloses, and very satisfactory products have been obtained from a number of materials, both woods and straws. To obtain any quantity of cellulose by the Cross and Bevan procedure is a slow and laborious business in that the amount which can be chlorinated at any one time is limited. By the new method 200–300 g. can be treated at a time.

The determination of lignin in cellulose products.

One of the vital requirements in a method of determination of cellulose is, of course, that lignin be completely removed. Hawley and Wise [1926] state that small amounts of lignin are often tenaciously retained by the Cross and Bevan cellulose product, yet it is surprising that cellulose methods have rarely been subjected to this ultimate test, namely the lignin content of the product. Ritter and Fleck [1924] compare figures for lignin in celluloses treated with chlorine for long and short periods. They show that with the longer treatments the lignin remaining is only slightly lessened, but they fail to comment on the significance of the lignin figures themselves. Chlorinations were continued till no colour was given on addition of sulphite, but even so the lignin content varied from 1·0 to 1·5 %. In this work a lignin determination was carried out on every cellulose product, since the appearance is not a satisfactory criterion.

Preparations of excellent colour may nevertheless contain appreciable quantities of lignin. This is very true of products obtained by the Jenkins method in which delignification is incomplete. In Table IX is given the lignin content of products obtained from straw by both the Cross and Bevan and the new procedure, continued until no colour is given with sulphite. The method adopted was to add to the cellulosic material 20 cc. 72 % sulphuric acid, and after repeated stirring to allow the whole to stand overnight in a cool place, the temperature not being allowed to exceed 20°. After dilution with 250 cc. distilled water, the solution was boiled for half an hour, allowed to settle and filtered. The longer period with 72 % acid and short hydrolysis at an acid concentration of about 9·5 % is preferred to the present method advocated by the U.S. Forest Products Laboratory, which involves only a brief treatment with strong acid and 4 hours' hydrolysis after dilution to an acid concentration of 3 %. By the method adopted the Cross and Bevan celluloses from straws contained approximately 3 % lignin, and this figure could not be lowered appreciably by overchlorination. Celluloses prepared by the new method were uniformly a little lower, but, even so, averaged about 2·5 %. Inasmuch as in both cases the wood celluloses were very considerably lower in lignin content it seemed that this high lignin figure might be apparent rather than real, and the reason for it inherent in the nature of the straw.

Recently a number of investigators [*e.g.* Wise and Fairbrother, 1931; Sherrard and Harris, 1932] have pointed out some of the defects of the 72 % sulphuric acid method for lignin, and some have made diametrically opposed recommendations as to the conditions under which the determination should be carried out. Ritter, Seborg and Mitchell [1932] suggest a brief treatment at 20°, while Peterson, Walde and Hixon [1932] prefer lower temperatures for a longer period and recommend that the reaction should be carried out in an ice-box. All are agreed that acid of this strength causes a certain amount of charring or caramelisation of the carbohydrate material, and that this may produce serious errors if the temperature rises above about 20°. Hawley and Wiertelak [1931] and Hawley and Harris [1932] have shown that a lignin-like material may be produced by exposure of cellulose or wood to a temperature of 135° for several days. Of the carbohydrate material concerned, the cellulosan, which is pentose in nature in the hardwoods and largely hexose in softwoods, suffers the greatest loss. Since, by a violent process of dehydration by heat, apparent lignin can be obtained in considerable amount from the cellulosans, it seemed possible by analogy that smaller quantities of some similar material might be formed by the action of 72 % sulphuric acid as used in the lignin determination. In some work, as yet unpublished, Norman has found that the cellulosans are readily removable by dilute acid hydrolysis. Lignin presumably is unaffected by dilute acid, since prolonged boiling in acid concentration of 4 % or more is generally accepted as a part of the estimation procedure. If, then, this theory is correct, the apparent lignin content of a cellulose from which the cellulosan has been removed by hydrolysis would be reduced as a result of the treatment. A sample of oat-straw cellulose prepared by the proposed new method had an apparent ash-free lignin content of 3·47 %. On boiling for 1 hour with 3 % H_2SO_4, a treatment known to remove a part, but not all, of the cellulosan, the cellulose suffered a loss of 17·1 % and the product had then an apparent lignin content (on the basis of the original cellulose present) of 2·37 %. Similarly a sample of oat-straw cellulose, prepared by the Cross and Bevan process and containing 3·0 % apparent lignin before hydrolysis, gave under the same treatment a product containing only 2·23 %. The effect of the removal of part of the cellulosan in both cases therefore was to lower the apparent lignin content by nearly a third. It seems clear that from the pentose material some product is being produced insoluble in boiling dilute acid and therefore determined as lignin. The 72 % sulphuric acid solution of the cellulose after removal of cellulosan was, incidentally, very markedly lighter in colour than that of the untreated material. The effect of hydrolyses with different strengths of acid for various times in lowering the apparent lignin content was next studied. Cellulose was prepared from wheat straw by the hypochlorite method, two neutral and three acid treatments being given. The product had an apparent lignin content of 2·4 %. 1·5 g. samples were hydrolysed under several conditions, filtered, dried and weighed and the lignin content of the residues then determined. The results are given in Table X. Treatment with 10 % acid, which, of course, results in appreciable hydrolysis of the cellulose itself as well as complete removal of the cellulosans, lowered the apparent lignin figure by a third to 1·6 %. Since this is still an amount higher than that found for most woods, it seemed desirable to investigate the nature of this residual apparent lignin, and to attempt to decide whether it is true lignin or secondary material produced in the course of the reaction. If true lignin, then it ought, perhaps, to be removable by gross over-chlorination and repeated extractions, whereas, if secondary material, such violent treatments should leave the amount very little changed.

Table X. *Effect of hydrolysis of cellulosans on the apparent lignin content of a wheat-straw cellulose containing 2·4 % lignin.*

H_2SO_4 strength %	Time in hours	Loss %	Furfurald. in solution %	Apparent lignin %
1·0	½	15·5	5·52	2·2
3·0	1	23·6	—	1·9
3·0	2	24·8	—	2·1
3·0	3	25·8	10·44	1·8
5·0	1	25·2	—	1·7
5·0	2	27·2	10·52	1·9
10·0	3	35·3	12·36	1·6

Wheat straw of apparent lignin content 20·3 % was treated in a series of 1·5 g. samples by the new method for cellulose determination. All the samples were given 2 neutral hypochlorite treatments, and then in pairs 1, 2, 3, 5 and 7 acid hypochlorite treatments respectively, each being followed by sulphite boiling. The apparent lignin was determined directly in one of the two duplicates and in the other after hydrolysis for 1 hour with 5 % H_2SO_4 to remove the cellulosan, as described above. The results are presented in Table XI. It will be seen that the apparent lignin content is appreciably lowered in all cases but the last by removal of the cellulosan. Even on gross over-chlorination to the extent of five extra acid hypochlorite treatments after the last trace of reaction with sulphite, the apparent lignin is quite appreciable, amounting to about 1·5 % on the original straw basis. Since it might have been supposed that these continued chlorinations would remove any residual lignin, this residue might be ascribed to material secondarily produced from the cellulose itself by charring or caramelisation. This view, however, is not supported by evidence from other materials. Results obtained with wood celluloses are of a considerably lower order, and both hardwood and softwood cellulose products have been obtained, as shown in Table IX, with an apparent lignin content as low as 0·1 to 0·5 % on the basis of the cellulose. Other hexosan material, such as

Table XI. *Removal of apparent lignin from wheat straw at successive stages of chlorination.*

All results expressed on original dry straw. (Apparent lignin = 20·3 %.)

Treatment	Colour reaction with sulphite	Product on chlorination %	Residue after hydrolysis with 5 % H_2SO_4 %	Apparent lignin in chlorination product	Apparent lignin in hydrolysis residue
—	—	—	57·23	—	15·2
2 N.	—	68·56	48·04	8·3	6·7
2 N. 1 A.	Strong	54·91	42·81	3·3	2·1
2 N. 2 A.	Trace	53·03	41·12	3·1	1·7
2 N. 3 A.	Nil	51·45	41·02	3·2	2·0
2 N. 5 A.	Nil	48·73	41·02	3·0	1·9
2 N. 7 A.	Nil	48·03	40·84	1·5	1·7

pure filter-paper or starch similarly yield only a trace of apparent lignin. It is not natural to suppose that straw cellulose is more susceptible to charring or caramelisation than other celluloses, since the magnitude of the apparent lignin figures is so different. The residue obtained, therefore, must be either lignin so tenaciously retained as to be unaffected by chlorination and sulphite extraction or some other acid-resistant constituent of unknown composition. This point will be tested further and an attempt made to characterise or extract this material by some such means as a modification of the glycol method of Hibbert and Rowley [1930].

Additional support for the view that from pentose material there is produced some material with the acid-resistant properties of lignin was obtained by carrying out that determination on filter-paper alone, filter-paper *plus* glucose and filter-paper *plus* xylose. 1 g. of filter-paper alone yielded 0·02 mg. apparent lignin, with the addition of an equal weight of glucose 0·03 mg. apparent lignin, but with the addition of only 0·25 g. xylose as much as 5·5 mg. apparent lignin.

Although somewhat outside the scope of this work the information obtained on the formation of apparent lignin from pentosan groupings may be of great importance in the ordinary determination of lignin in untreated materials. W. G. Campbell in a private communication has expressed the opinion that the hemicelluloses are a source of error in the lignin determination. It may therefore be necessary to modify the method by the introduction of a mild hydrolytic treatment for the removal of these constituents. In Table II is shown the effect of a brief extraction of oat straw with HCl, the apparent lignin content

Table XII. *Effect on apparent lignin content* of preliminary hydrolysis of straws and woods (5 °/₀ H_2SO_4 for 1 hour).*

Results expressed on 100 g. material.

Straw	Apparent lignin %	Loss on hydrolysis %	Apparent lignin after hydrolysis
Oats	18·4	42·5	12·6
Wheat	19·3	37·5	13·7
Barley	15·7	49·4	11·2
Beech†	22·8	26·7	19·2
Scotch pine†	24·1	23·6	23·7

* Determined by treatment for 16 hours with 72 % H_2SO_4, dilution to 3 % and boiling for 2 hours. These figures therefore are directly comparable with those obtained by other workers.

† Results expressed on material extracted with alcohol-benzene.

falling from 19·9 to 13·5 %, a loss of no less than a third as a result of a treatment which would presumably not affect true lignin. Similarly some of the straws and woods employed were boiled for 1 hour with 5 % sulphuric acid and almost equally large losses in apparent lignin content noted, as shown in Table XII.

These great differences make it clear that the whole question of the determination of lignin by the 72 % sulphuric acid method must be critically re-examined, since, if the indications given here are correct, the quoted lignin figures for straws and hardwoods are too high as a result of secondarily produced material from the pentose groupings. Those for the softwoods, in view of the smaller content of pentose material, are probably more nearly accurate. This may be seen from the figures given for beech and Scotch pine in Table XII.

SUMMARY.

1. The cellulosans or polysaccharide material very intimately associated with "pure" cellulose in nearly all natural celluloses should remain intact in any satisfactory method of cellulose estimation.

2. Alkaline pre-treatment, as often recommended, is objectionable in that a portion of the cellulosan is removed, even though delignification is much assisted.

3. Jenkins's method of cellulose determination is unsatisfactory for two reasons. First, the preliminary treatments with alkali and acid alternately result in a very severe loss of cellulosan, and second, the product is incompletely delignified by the subsequent treatments with neutral hypochlorite.

4. In the chlorination methods of cellulose determination the chlorine is at least as important in causing the solution of hemicelluloses during the subsequent treatment with boiling sulphite as in assisting in the removal of lignin. Sulphite alone results in extensive, but not complete, delignification without heavy loss of encrusting polyuronide hemicelluloses.

5. Neutral hypochlorite treatments followed by sulphite boilings will effect a fairly complete delignification of plant materials.

6. A new method is proposed for both straws and woods involving first two treatments with neutral hypochlorite and then three or more with acid hypochlorite, each followed by boiling with sodium sulphite. The results obtained are almost identical with those obtained by the Cross and Bevan method without pre-treatment. The proposed method is more rapid with straws in that it involves fewer treatments. It is readily applicable to large scale preparations.

7. The products from cereal straws given by both the Cross and Bevan and the new methods are not entirely free from lignin. The lignin figure obtained by the use of 72 % H_2SO_4 on such products is, however, not accurate, since appreciable quantities of apparent lignin are produced from the cellulosan when pentose in nature. Upon removal of this fraction by acid hydrolysis the lignin figures are reduced by a quarter to a third, the residue being unaffected by over-chlorination. It must therefore be either true lignin very tenaciously held, or else some other resistant material of unknown composition.

8. In view of the production of apparent lignin from pentosan groupings by 72 % H_2SO_4, the figures obtained for lignin on many natural materials may be unreliable. A brief preliminary acid hydrolysis of straws results in the lowering of the apparent lignin content by 25–30 %. Hardwoods similarly are likely to give figures which are too high.

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CIX. METABOLISM OF NORMAL AND TUMOUR TISSUE.

VIII. RESPIRATION IN FRUCTOSE AND IN SUGAR-FREE MEDIA.

By FRANK DICKENS and GUY DRUMMOND GREVILLE.

From the Courtauld Institute of Biochemistry, the Middlesex Hospital, London, W. 1.

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THE aim of the work presented in this series of publications is to determine the nature of the foodstuffs utilised by various types of surviving animal tissue and the proportions in which they are consumed. To this end, in Parts II and IV [Dickens and Šimer, 1930, 2; 1931, 2] the tissues were classified according to the magnitude of their respiratory quotients in glucose-containing media; but the complete interpretation of these quotients requires far more data than are at present available. For example, when the R.Q. approaches unity, preponderating carbohydrate oxidation may not be assumed, unless protein oxidation is known to be slight; for pure protein oxidation ending in ammonia production causes the R.Q. to approach unity. Again, lowered R.Q. is commonly regarded as an indication of fat oxidation, though direct proof of this with surviving tissue is lacking: here also protein oxidation would lead to uncertainty, for when the end-product is urea the R.Q. is about 0·8. Complete analysis of the R.Q. thus demands proof of the utilisation of the different foodstuffs and a study of the nature of the end-products. Work is proceeding with these ends in view, and we hope shortly to publish an account of ammonia and urea formation under our experimental conditions.

Evidence for the consumption of carbohydrate by a given tissue may be obtained in the absence of the extensive data required for the complete understanding of the R.Q. The interpretation of chemical estimations of sugar disappearance may be difficult, but disappearance of glycolysis under aerobic conditions, or prevention of ammonia formation by the presence of sugar suggests carbohydrate oxidation. Again, if the R.Q. and the oxygen uptake fall in the absence of sugar, carbohydrate oxidation may reasonably be inferred. In the present paper, by a systematic study of the oxidative metabolism of the various tissues in the absence of added substrate, we attempt to decide which tissues are able to oxidise glucose. In addition, we have studied the oxidation of fructose by these tissues. Fructose is of interest because as previously shown [Dickens and Greville, 1932, 1, 2] unsuspected differences in anaerobic lactic acid formation are revealed by the use of fructose as substrate; and further because the peculiar oxidation of fructose by brain led Loebel [1925] to the view that lactic acid is probably not an intermediate product in the complete oxidation of hexoses. It is therefore of peculiar interest to compare wherever possible the anaerobic fructolysis of the various tissues with their carbohydrate oxidation in the presence of fructose and to calculate the Meyerhof quotient where fructose is the substrate.

EXPERIMENTAL.

Methods.

Measurements were made by the methods of Dickens and Šimer in phosphate media [1930, 1] and in bicarbonate media [1931, 1]. These are referred to in the tables as P and B respectively. In addition, some measurements in bicarbonate were made by Warburg's two-vessel method [Warburg, 1924] (B* in the tables). The media were as previously described, but contained, in addition to Ca and K, magnesium chloride in 0·00087 molar concentration. Unless it is stated otherwise, in the phosphate experiments the tissue was washed for 5 to 10 minutes before the experiment by suspending it at room temperature in sugar-free phosphate Ringer solution through which a stream of oxygen was passed, and in the bicarbonate experiments the tissue was rinsed under the same conditions in sugar-free bicarbonate Ringer solution in a stream of oxygen containing 5 % CO_2. For embryo and retina warmed Ringer solution was used; the latter tissue was merely rinsed in several changes of oxygenated Ringer solution, and the preparation and determinations were carried out in a dim red light. Anaerobic measurements of glycolysis were all made by Warburg's method in bicarbonate-containing Ringer solutions. The tissues used were from fed animals.

Results.

The results are given in Tables II, III and IV for adult normal, embryonic and tumour tissues respectively. For the sake of simplicity the data are summarised in Table I, where for several tissues the mean values from the other tables are given, but in other cases, when the number of observations is less, rounded figures are inserted. In deriving the figures in this table for respiration in glucose and for the anaerobic glycolysis, consideration has been given to results already published in this series.

Respiration with and without glucose. The nervous tissues, brain cortex and retina, possessing a R.Q. of unity, are markedly dependent on the presence of glucose both for their respiration and for their anaerobic glycolysis. It is interesting to note that in our experiments both with rat and with rabbit brains a R.Q. of about 1 is maintained with the greatly diminished oxygen uptake in the absence of added substrate. It seems unlikely that when deprived of hexose the brain can change its type of metabolism to that of fat oxidation. Possibly some of the oxygen uptake under these conditions is due to protein oxidation [Loebel, 1925] although alanine is neither oxidised [Loebel, 1925] nor deaminated [Krebs, 1932] by this tissue. It will be seen that for the maintenance of its respiration retina is even more dependent than brain on its supply of sugar (Fig. 1); here, however, the low respiration and the small amount of retina available make the determination of R.Q. in the absence of substrate almost impracticable.

In contrast to the nervous tissues, kidney, liver and spleen suffer relatively little fall in respiration when deprived of glucose [compare Meyerhof and Lohmann, 1926, 1]. In kidney, the respiration is fairly constant in the presence of glucose, but falls off appreciably with time in its absence (Fig. 3). Q_{O_2} is remarkably constant in either medium from one experiment to the next (Table II), and the mean values agree remarkably well with those given by Meyerhof and Lohmann. The R.Q. is definitely raised by the presence of glucose. In spleen, although Q_{O_2} is little increased by sugar, evidence of glucose consumption is provided by the raised R.Q. In liver, the R.Q. and Q_{O_2} appear to be independent

Table I. *Summarised results.*

Tissue	Sugar	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Adult normal tissues:					
Brain cortex (rat)	Glucose	1·01	12·8	1·5	11·5
	Fructose	0·99	9·7	0·3	1
	None	1·00	3·9	−0·4	0·5
Brain cortex (rabbit)	Glucose	—	7·5	1·5	12
	Fructose	1·01	8·5	0·5	3
	None	1·02	5	−1	—
Retina (rat)	Glucose	1·00	22	22·5	64
	Fructose	1·01	16	0·3	3·5
	None	—	4	—	3·5
Testis (rat)	Glucose	0·88	11·2	7·2	12
	Fructose	0·82	9·3	2·7	4
	None	0·6–0·8	5·2	1·9	2
Kidney (rat)	Glucose	0·85	20·6	0	6
	Fructose	0·90	25·5	0·2	2
	None	0·80	17·6	−1	1
Spleen (rat)	Glucose	0·91	11	5	8
	Fructose	0·83	11	4	3
	None	0·83	9	0·5	3
Liver (rat)	Glucose	0·68	7	3	1·5
	Fructose	0·78	7·5	3	4
	None	0·66	7·8	2	1·5
Embryonic tissues:					
Chick embryo (5 day)	Glucose	1·00	10	2	15
	Fructose	0·92	9·3	0	1·5
	None	0·89	8·3	0	1·5
Rat embryo (13–14 day)	Glucose	1·00	11	—	12
	Fructose	0·98	11·5	0	3
	None	0·7–1	9	0·5	2
Rabbit embryo	Glucose	1·00	8·5	—	13
	Fructose	1·00	7	—	2
	None	1·00	6·5	—	—
Rat yolk-sac	Glucose	1·00	11	2	20
	Fructose	0·98	10	1	15
	None	0·93	11·5	0	2
Tumour tissue:					
Jensen rat sarcoma	*Glucose	0·84	9·6	11	31
	*Fructose	0·84	11·1	7	13
	*None	0·80	10·0	0	2
	†Glucose	—	11	20	30
	†Fructose	—	10	7	10

* Methods of Dickens and Šimer [1930, 1; 1931, 1]. † Method of Warburg [1924].

of glucose, at least in the fed animals used in our experiments. The portion of the oxidation covered by carbohydrate in liver is presumably to be referred to the oxidation of the glycogen reserves [Takane, 1926]: by a preliminary aerobiosis these may be induced to yield a large anaerobic glycolysis, the magnitude of which is quite unaffected by the presence of glucose [Rosenthal, 1929; 1931]. There is, therefore, no evidence at present that glucose can be utilised either aerobically or anaerobically by isolated post-adsorptive liver tissue.

Intermediate between these tissues and the nervous tissues, testis shows a large decrease in respiration when deprived of glucose (Fig. 2), the R.Q. at the same time falling by a variable amount. It is assumed from the R.Q. that these four tissues, liver, kidney, spleen and testis, possess a mixed fat-carbohydrate metabolism. It may further be assumed that liver, spleen and kidney have

Table II. *Adult normal tissues.*

Exp. No.	†Medium	Time hrs.	Glucose				Fructose				No addition			
			R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Brain cortex (rat):														
1	P	2	1·01	12·8	—	—	1·03	10·9	—	—	1·04	4·0	—	—
2	B	3	1·00	12·8	1·9	—	1·01	9·0	0·5	—	—	—	—	—
3	B	3	—	—	—	—	0·97	9·3	0·0	—	—	—	—	—
4	B	2½	1·01	12·5	1·0	—	—	—	—	—	0·96	3·7	−0·4	—
											0·96	3·7	−0·4	—
Brain cortex (rabbit):														
5	B*	3	—	7·6	1·5	12·0	—	9·2	0·6	3·2	—	—	—	—
6	B	3	—	—	—	—	1·01	7·9	0·7	3·3	—	—	—	—
7	B	2½	—	—	—	—	—	—	—	—	1·02	4·8	−1·2	—
Retina (rat):														
8	P	5	1·00	24·4	—	—	1·02	14·6	—	—	—	4·5	—	—
							0·99	16·2	—	—	—	3·0	—	—
9	B	5	—	20·3	22·5	—	—	17·2	0·3	—	—	—	—	—
10	B	1	—	—	—	64·0	—	—	—	3·5	—	—	—	3·5
Rat testis:														
11	P	3	—	—	—	—	0·83	8·8	—	—	0·57	5·5	—	—
	B	3	0·87	10·2	6·0	—	—	—	—	—	0·57	4·6	2·2	—
12	P	3	0·89	12·6	—	—	0·87	9·0	—	—	0·82	6·2	—	—
	B	3	0·88	10·2	6·8	—	0·82	8·5	2·1	—	0·82	4·8	1·8	—
13	B	2½	0·87	13·0	9·0	11·8	0·76	11·6	3·8	4·0	—	—	—	1·6
14	B	3	0·88	10·2	6·8	—	0·82	8·5	2·1	—	0·82	4·8	1·8	—
Mean			**0·88**	**11·2**	**7·2**	**11·8**	**0·82**	**9·3**	**2·7**	**4·0**	—	**5·2**	**1·9**	—
Rat kidney:														
15	P	2	0·82	21·5	—	3·5	0·92	24·0	—	2·4	0·80	16·5	—	1·3
16	P	2½	0·85	21·9	—	4·6	0·92	26·1	—	2·5	0·80	15·8	—	2·0
17	B	2½	0·85	18·0	0·0	8·0	0·88	25·1	0·4	0·7	0·81	17·8	−1·3	0·2
18	B	3	0·84	19·3	0·0	—	0·91	27·6	0·1	—	0·80	19·0	−1·1	—
18 A	B	3	0·87	22·2	2·0	7·6	0·89	24·9	0·2	2·8	0·79	18·9	−0·6	1·3
Mean			**0·85**	**20·6**	—	—	**0·90**	**25·5**	—	—	**0·80**	**17·6**	—	—
Rat spleen:														
19	B	3	0·92	10·8	4·8	—	0·81	10·5	3·6	—	0·83	8·9	0·3	—
20	B	3	0·89	11·0	5·3	—	0·85	12·3	4·0	5·6	0·83	9·0	0·6	4·4
Rat liver:														
21	P	3	0·69	6·7	—	—	0·77	7·0	—	—	0·71	7·1	—	—
22	B	4	—	—	—	—	0·77	7·4	3·5	4·0	0·70	7·8	2·0	1·4
							0·79	8·5	2·9	—	—	—	—	—
23	B	3	0·67	7·3	2·8	1·7	0·78	7·0	3·0	3·6	0·58	8·6	2·1	1·5

† For aerobic experiments. P = Phosphate. R.Q. determination by method of Dickens and Šimer (1930, 1]. B = Bicarbonate: method of Dickens and Šimer [1931, 1]. B* = Bicarbonate: method of Warburg [1924].

a foodstuff supply not available to testis: with liver this is definitely glycogen, but of its nature in spleen and kidney we cannot at present be sure.

The embryonic tissues studied (chick, rat and rabbit embryos; rat and rabbit yolk-sac; ferret chorion) have, like the nervous tissues, a high anaerobic glycolysis coupled with a relatively small autoglycolysis and resemble them further in possessing a R.Q. of unity in glucose. The strong falling off in respiration in the absence of glucose which might be expected by analogy does not, however, occur. Although deprivation of glucose usually produces a marked fall in R.Q., the decrease in oxygen consumption is either small, or, as with the rat yolk-sac, does not occur at all. How much of the respiration in absence of sugar is due to oxidation of preformed lactate in the tissue is uncertain. However, with the exception of the experiments marked (*a*) in Table III, the washing of the tissue before the experiment was at least as good as that of brain and testis, where the respiration falls heavily in the absence of substrate. In the two experiments marked (*b*), the tissue was shaken in the bath in phosphate Ringer saturated with oxygen for 10 minutes before the experiment, and this efficient washing

Table III. *Embryonic tissues.*

Exp. No.	Medium	Time hrs.	Glucose				Fructose				No addition			
			R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
Chick embryo (5 days' incubation):														
34[a]	P	4	1·02	10·3	—	—	0·96	9·7	—	—	0·94	8·2	—	–
35[b]	P	3	—	—	—	—	0·96	9·3	—	—	0·87	9·2	—	–
36[b]	P	4¼	—	—	—	—	0·87	9·6	—	—	0·89	8·3	—	–
37[a]	B	3½	1·02	9·1	2·4	—	0·89	8·5	0·0	—	0·87	7·5	0·0	–
38[a]	B	4	—	—	—	—	0·94	8·1	−0·6	—	0·91	8·0	−0·7	–
39[a]	B	4	—	—	—	—	0·85	10·4	0·4	—	0·84	8·7	0·3	–
Mean			[1·00]	—	—	—	**0·92**	**9·3**	—	—	**0·89**	**8·3**	—	–
Rat embryo (13–14 days):														
40[c]	P	3	—	—	—	—	1·01	10·9	—	3·1	0·70	7·6	—	1·9
41[d]	P	3	1·00	10·9	—	—	0·99	12·3	—	3·1	0·97	10·1	—	2·1
42[e]	B	3	0·97	11·1	5·2	—	0·95	11·4	0·0	—	0·78	9·2	0·7	0·7
Rabbit embryo:														
43[f]	P	3	1·00	8·6	—	13·3	1·00	7·1	—	1·8	1·00	6·4	—	–
Rat yolk-sac:														
44[g]	P	1st hour	—	14·8	—	18·0	—	12·9	—	11·5	—	14·0	—	4·0
		2nd hour	—	12·9	—	17·0	—	11·9	—	9·0	—	12·9	—	1·0
		3rd hour	—	13·2	—	17·5	—	11·9	—	8·5	—	13·2	—	1·0
45[h]	B	4	—	—	—	—	0·98	11·0	1·1	—	0·91	11·4	0·3	–
46[i]	B	4	1·04	12·0	2·3	—	—	—	—	—	0·94	11·8	−0·4	–
											0·94	11·9	0·0	–
47[g]	B*	1	[1]	8·7	0·3	20·0	[1]	8·0	0·5	21·0	—	—	—	2·5
48	B	3	—	—	—	—	—	—	—	—	0·92	8·9	1·1	–
Rabbit yolk-sac (28 days):														
49	P	3	0·97	7·5	—	—	—	—	—	—	0·81	8·4	—	–
Ferret chorion (6 g. embryo):														
50	P	3	0·99	6·7	—	17·5	—	—	—	—	0·88	5·1	—	2·0

[a] Rinsed in salt solution in dish.
[b] Well washed by shaking 10 mins. in Ringer solution.
[c] 5·3 mg. dry weight.
[d] 3·9 mg. dry weight.
[e] 6·8 mg. dry weight.
[f] 12·2 mg. dry weight.
[g] 20 day embryo.
[h] 19 day embryo.
[i] 18 day embryo.

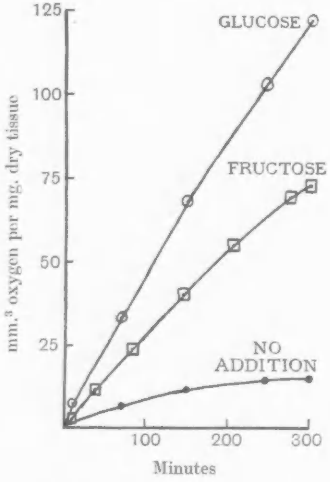

Fig. 1. Respiration of rat retina.

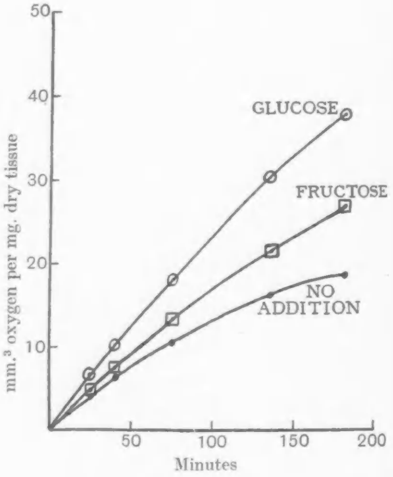

Fig. 2. Respiration of rat testis.

does not reduce the respiration in absence of sugar below the values obtained with the less efficient washing of the experiments (*a*). Further, the lactate consumption necessary to maintain this Q_{O_2} should be indicated by a large negative $Q_M^{O_2}$ which is certainly not observed. Taking the rat yolk-sac as example, we see from Exp. 44 that in the second and third hours the autoglycolysis would account for less than one-third of the respiration; the remainder, if due to lactate oxidation, would demand a negative $Q_M^{O_2}$ of about 3.

Table IV. *Tumour tissue: Jensen rat sarcoma.*

			Glucose				Fructose				No addition			
Exp. No.	Medium	Time hrs.	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	R.Q.	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$
24	P	2½	0·85	10·6	—	—	0·87	13·1	—	—	0·81	12·8	—	—
25	P	3	0·91	7·3	—	22	0·91	8·0	—	14·0	0·87	7·0	—	3·5
26	P	3	0·85	6·7	—	—	0·85	9·6	—	5·9	0·78	8·0	—	—
27	B	3	0·86	11·3	9·0	33	0·80	12·0	7·2	17·0	0·82	10·7	−0·7	1·5
28	B	3	0·80	11·2	11·2	40	0·88	10·6	6·4	15·1	—	—	—	—
29	B	2½	0·83	9·3	9·3	—	—	—	—	—	0·73	9·1	0·7	—
30	B	3	—	—	—	—	0·81	14·1	5·6	—	0·78	12·6	0·1	—
31	B	2½	0·77	11·1	11·8	27·5	0·77	10·8	10·2	10·8	—	—	—	1·7
							0·76	10·1	8·9	—	—	—	—	—
							0·74	11·0	11·2	—	—	—	—	—
Mean			**0·84**	**9·6**	—	—	**0·84**	**11·1**	—	—	**0·80**	**10·0**	—	—
32	B*	2	[0·78]	10·3	25·4	32·0	[0·78]	11·8	8·3	11·5	—	—	—	—
33	B*	2	[0·8]	11·7	15·4	28·0	[0·8]	8·7	6·0	9·3	—	—	—	3·0

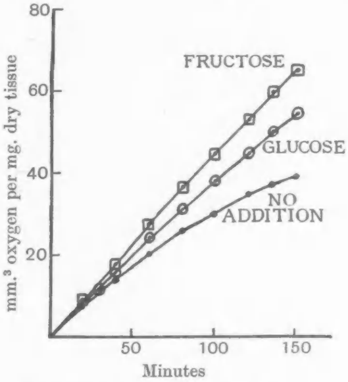

Fig. 3. Respiration of rat kidney.

As acid consumption of such magnitude was nowhere observed in these experiments, we may assume that the maintenance of respiration in the absence of sugar is not due to consumption of preformed lactate. It is also unlikely that it is largely due to protein oxidation leading to urea or ammonia. From experiments to be reported later, it appears that the urea formation by the 5 day chick embryo and the late rat yolk-sac in the absence of sugar is negligible. In the former case ammonia was formed, which, if due to pure protein oxidation, might account in some embryos for rather less than half the respiration: in the latter, protein oxidation leading to ammonia is apparently inconsiderable. We therefore consider that the rat yolk-sac and the chick and rat embryos possess reserve foodstuffs of whose nature we are ignorant.

The Jensen rat sarcoma shows a surprisingly small fall in R.Q. when it is deprived of glucose. Crabtree [1929] found that the respiration of the Jensen rat sarcoma under experimental conditions different from ours was lower in the presence of glucose than in its absence, but our experiments give little support to this finding.

Oxidative metabolism of fructose. As fructose undergoes autoxidation in strong neutral phosphate solutions [Warburg and Yabusoe, 1924], a control experiment was performed on the oxygen uptake of the fructose-containing Ringer solutions used in the method for the determination of R.Q. in phosphate media. The pressure change in 3 hours lay within the error of experiment.

It was shown by Loebel [1925] that the rat brain cortex, although unable to convert fructose into lactic acid, could nevertheless oxidise it almost as readily as glucose, and with a R.Q. of unity. This finding is fully confirmed by the results shown in Table II, not only in phosphate media, but also in bicarbonate media where in our experiments the aerobic and anaerobic measurements were made under almost identical conditions of p_H and bicarbonate content. It is interesting to note the effect on the value of the Meyerhof quotient. In Exp. 5 with rabbit brain, the respiration experiment was performed by Warburg's two-vessel method, and the initial p_H in both aerobic and anaerobic experiments was 7·5. The calculation of the Meyerhof quotient for glucose and fructose is given below:

Sugar	Hour	Q_{O_2}	$Q_M^{O_2}$	$Q_M^{N_2}$	$Q_M^{N_2}-Q_M^{O_2}$	Meyerhof quotient
Fructose	1	9·2	1·4	6·3	4·9	0·53
	2	9·6	0·5	1·7	1·2	0·13
	3	8·7	0·1	1·5	1·4	0·16
Glucose	1	8·2	2·8	16·0	13·2	1·6
	2	7·3	0·9	10·6	9·7	1·3
	3	7·2	0·7	9·3	8·6	1·2

It appears that the view that fructose is directly oxidised by brain without conversion into lactic acid is correct. If we add to the respiration in absence of sugar the amount of respiration which could be supported by the difference between the fructolysis and the autoglycolysis, then both in Loebel's experiments and in ours the resulting value of Q_{O_2} falls far short of the Q_{O_2} attained in fructose. Again, if the anaerobic fructolysis of brain could be raised, like the autoglycolysis of liver, by a preliminary aerobiosis [Rosenthal and Lasnitzki, 1928], it might be that aerobic conditions would render brain tissue capable of converting fructose into lactic acid preparatory to its oxidation. We have, however, found that a 30 minutes' preliminary aerobiosis produced no increase in the anaerobic fructolysis of brain. We may therefore conclude that fructose is indeed directly oxidised by brain cortex.

Another clear case of direct oxidation of fructose is provided by retina (Fig. 1). This tissue, which resembles brain cortex particularly in its dependence on sugar for the maintenance of its respiration and also in its inability to convert fructose into lactic acid, is now seen to resemble brain cortex further in its ability to oxidise this sugar directly (see Table II). Meyerhof and Lohmann [1926, 1] showed that the aerobic acid production by brain cortex in glucose disappeared when fructose was the substrate: this is seen more dramatically in retina (Table II), where the large aerobic glycolysis characteristic of this tissue is replaced by a negligible fructolysis.

Testis shows a slight tendency to convert fructose into lactic acid [Dickens and Greville, 1932, 2]. The strong falling off in respiration of this tissue in the absence of sugar is prevented by fructose (Fig. 2), although R.Q. and Q_{O_2} do not reach the values they attain in glucose. Liver, as shown by Rosenthal, possesses a variable but often strong fructolysis. It is therefore not surprising that the R.Q. is considerably raised by this sugar. It is remarkable, however, that the respiration is not influenced; for it may be noted that a large increase in Q_{O_2} is produced by replacing glucose by lactate in pre-absorptive liver [Meyerhof and Lohmann, 1926, 2] and this effect is still definite in post-absorptive liver [Dickens and Šimer, 1930, 2].

The respiratory data in the case of spleen do not show whether fructose is utilised. Since, however, the presence of fructose reduces the aerobic ammonia production by this tissue, as will be shown in a later publication, it is probable that fructose is oxidised to some extent.

Kidney provides an interesting case. Meyerhof and Lohmann [1926, 1] showed that fructose increased the respiration above the value in glucose. The increase shown in Table I agrees well with that found by these authors. We show that the R.Q. also is raised above the value in glucose. Since the anaerobic fructolysis of kidney is only one-third of the glucolysis, this would appear to be a case of direct oxidation. However, caution is necessary in this case. Rosenthal and Lasnitzki [1928] found that the anaerobic glycolysis could be accelerated about 50 % by preliminary aerobiosis. We have observed an "extra-fermentation" of the same order both in glucose and fructose. It is therefore not impossible that under optimum aerobic conditions kidney may be capable of forming far greater quantities of lactic acid than are shown by the anaerobic figures given in the tables. Nevertheless, it is difficult to explain why the kidney oxidises fructose more readily than glucose.

The aerobic utilisation of fructose by the various embryonic tissues is of interest in view of the remarkably varied power of converting this sugar into lactic acid which these tissues possess [Dickens and Greville, 1932, 1]. The rat yolk-sac has an anaerobic fructolysis approaching its glucolysis. The R.Q. is raised to unity by fructose, whilst Q_{O_2} is practically equal to that in glucose and in sugar-free solutions, and the aerobic fructolysis is small. Thus with fructose as well as glucose, the respiration is capable of removing almost all the glycolysis. The embryo does not convert fructose into lactic acid, and it is of interest to see whether it can oxidise it. The manometric data for the chick embryo do not give any definite information on this point, but the results of ammonia estimations suggest that some oxidation may occur. For the rat embryo the R.Q. measurements provide definite evidence of aerobic utilisation of fructose. Whilst the results with embryo are not striking, they at least show that fructose can diffuse into the tissue, and that the failure of the embryo to fructolyse is not due to an impermeability towards this sugar.

The R.Q. of the Jensen rat sarcoma is raised by fructose to the value attained in glucose. *d*-Lactate produces an increase in R.Q. above the value in sugar-free Ringer solution of the same order, as is shown by the following experiment:

	R.Q.	Q_{O_2}	$Q_M^{O_2}$
No addition	0·76	−10·9	0·0
0·2 % *d*-lactate	0·79	−12·7	−0·7

For the better study of the aerobic acid production in this tissue, Exps. 32 and 33 were performed by Warburg's method, as the Dickens-Šimer method for the determination of respiratory quotient in bicarbonate media gives much lower values of $Q_M^{O_2}$ with Jensen rat sarcoma than does the two-vessel method.

It will be seen that there is a considerable aerobic fructolysis: the reduction by oxygen of the acid production in fructose is remarkably small. Both fructose and glucose therefore seem to be capable of saturating the tissue with lactic acid, supplying it under aerobic conditions faster than it can be utilised. The lactate oxidation of Jensen rat sarcoma is insufficient; and there is no evidence that fructose can be directly oxidised by this tissue. The power to oxidise carbohydrates is clearly small, but we cannot as yet state what fraction of the total oxygen uptake is to be referred to carbohydrate oxidation. The failure of the respiration to fall off in the absence of sugar increases the difficulty in determining this fraction; but it is hoped that a study of the nitrogenous end-products will assist us to assess its magnitude.

SUMMARY.

1. The oxidative metabolism of surviving adult normal tissues (brain cortex, retina, testis, liver, kidney, spleen), embryonic tissues (rat yolk-sac, chick and mammalian embryos) and tumour tissue (Jensen rat sarcoma) has been studied in the absence of sugar and compared with that in the presence of glucose. Tissues of fed animals were used.

2. The nervous tissues (brain, retina) suffer a large fall in respiration when deprived of glucose. Kidney and spleen are less affected, but testis shows a marked dependence on the supply of sugar. As a result of the experiments, it seems probable that brain, retina, testis, kidney and spleen all oxidise glucose though to differing extents; but there is no evidence that the oxidative metabolism of post-adsorptive liver is affected by glucose.

3. The respiration of the embryonic tissues studied falls very little when they are deprived of glucose. They appear to contain reserve foodstuffs of whose nature we are ignorant.

4. The respiration of Jensen rat sarcoma is not decreased in the absence of glucose: the R.Q. falls slightly.

5. The oxidative metabolism of the same adult normal, embryonic and tumour tissues has been studied with fructose as substrate. It appears probable that all these tissues oxidise this sugar to a greater or less extent. Brain and retina oxidise it strongly, with a R.Q. of unity, probably without intermediate conversion into lactic acid. The R.Q. of testis, kidney and liver is raised by fructose above the value in the absence of sugar. Rat embryo and yolk-sac have a R.Q. of approximately 1 in its presence.

6. Jensen rat sarcoma has the same R.Q. in glucose and in fructose, and with this tissue in fructose as in glucose strong acid production persists in the presence of oxygen.

We wish to express our thanks to Prof. E. C. Dodds for his interest in these experiments.

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CX. ON CATATORULIN.

A NEW METHOD OF COMPARING THE OXIDATIVE FACTOR IN VITAMIN B_1 CONCENTRATES.

By REGINALD PASSMORE[1], RUDOLPH ALBERT PETERS
AND HUGH MACDONALD SINCLAIR[2].

From the Department of Biochemistry, Oxford.

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In recent work Gavrilescu, Meiklejohn, Passmore and Peters [1932] have shown that potent vitamin B_1 concentrates in very small amount increase the oxygen uptake of avitaminous pigeon's brain in lactate solutions. The effect is proved to be catalytic, because the extra oxygen taken up is far in excess of the amount of vitamin B_1 added. To avoid begging the question as to the identity of the oxidase factor in the concentrates with vitamin B_1, and to economise words, we propose to call it "catatorulin." There is very strong evidence to support the view that catatorulin and vitamin B_1 are identical. In particular Meiklejohn, Passmore and Peters [1932, 1, 2] found that the catatorulin effect was directly related to the well-known symptom of head retraction in pigeons, and that it disappeared with cure of the birds by vitamin. The indirect evidence in favour of identity is strengthened by the fact, presented here for the first time, that the crystalline preparations of Kinnersley, O'Brien and Peters [1933] exhibit catatorulin action. Nevertheless these workers are not yet convinced that the crystals represent pure vitamin, and therefore any finding of parallelism between catatorulin and vitamin B_1 preparations is of importance.

With these objects in view, a method has been devised for comparing the effects upon oxygen uptake of two different vitamin B_1 preparations. Avitaminous brain tissue must be used because the normal shows no effect with added vitamin B_1. Attempts to obtain stable dry preparations so far have failed.

There has proved to be good correspondence between vitamin B_1 and catatorulin in the crystalline preparations; the method therefore promises to be of value as a new means of estimating the activity of potent vitamin B_1 preparations. It has the great advantage that bird variations are eliminated, and that a comparison is made upon an enzyme system rather than upon the whole animal.

Experimental.

The brains of polyneuritic birds showing well-marked head retraction must be used; the birds were fed by the usual method employed in this laboratory. It is an advantage to use animals which have already been under test, as the brains seem more generally deficient in the vitamin. After guillotining (best at the neck alone to avoid injury to the tissue), the blood is allowed to drain for

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[1] Senior Scholar of Brasenose College.

[2] Senior Demy of Magdalen College.

1 minute from the neck; the brain is then uncovered, mopped lightly with filter-paper and removed; all parts except the cerebellum are finely minced with a bone spatula[1] and thoroughly mixed.

The cerebellum has been excluded owing to previous findings, which have not been further investigated. It would have been better if one part of the brain could have been selected, but this does not provide enough tissue for comparison.

Samples of approximately 100 mg. (up to 16 in number) are introduced into tared and corked bottles of Barcroft-Dixon apparatus, previously filled with Ringer-phosphate lactate solution (p_H 7·3)[2]. The weight is determined by a second weighing.

The Ringer solution used has the composition NaCl 0·9 %, KCl 0·025 %, $CaCl_2$ 0·030 %, $NaHCO_3$ 0·015 %. To 786 cc. Ringer solution 200 cc. of KH_2PO_4 6·8 % are added, and then 13·9 cc. 20 % NaOH solution to bring to p_H 7·3. After standing for one day, the precipitate is removed by filtration. To each 49 cc. of the Ringer-phosphate is added 1 cc. of a neutralised Na lactate solution, containing sufficient lactic acid to make the final concentration 0·033 *M*. The lactate solution was prepared from recrystallised zinc lactate, as formerly described. It is convenient to neutralise, make up so that the required amount of lactate is present in 1 cc. solution, and then to introduce 1 cc. amounts into sterile tubes fitted with cotton-wool plugs. These can be sterilised on 3 successive days for 20 minutes in the steamer and kept in cold store. One tube serves for an experiment. Glass-distilled water is used throughout.

After the second weighing the tissue is finely broken up with a flat-ended glass rod, and immediately following this is added the vitamin made up in Ringer-phosphate lactate; the volume of this should be at least 0·1 cc., and allowance must be made when filling the bottles, so that the solution with additions reaches 3·0 cc. If care is taken not to remove more fluid than absolutely necessary with the glass rod, then any error from this cause will be negligible. The roll of filter-paper is now inserted, and wetted with 2*N* KOH, after which the bottles are twice evacuated[3] to 70 mm. and filled with oxygen, and the taps closed. Excessive evacuation seems to spoil the tissue. These operations take about 30 minutes from the time of guillotining. When evacuation is completed, the bottles are placed in the bath at 38°, the taps being opened temporarily to equalise pressure; they are shaken with several levellings for 9 minutes, and the taps finally closed. The first reading is taken 12 minutes after introducing into the bath, and thereafter at intervals of 30 minutes.

The temperature of 38° represents a compromise between the temperature of normal and avitaminous animals. Doubtless the experiments would be more efficient at 41°, but as the effect is well obtained at 38° we have adhered to this temperature, which makes all our observations comparable with those made earlier here.

Preparation of vitamin solutions.

The standard solutions (200γ crystals of vitamin B_1 per 1·0 cc. acid alcohol *N*/1000), as made for the other work in this Laboratory, were used for most of the work. We are indebted to Mr H. W. Kinnersley for his help in their preparation. The necessary amount of vitamin, 0·05–0·1 cc., was measured into a clean small test-tube, one drop of water added, and the alcohol removed on the water-bath. Immediately before addition to the bottles, Ringer-lactate phosphate solution was added together with an appropriate volume of *N*/1000 Na_2CO_3 to neutralise the acid. A convenient volume for addition was found to be 0·5 cc. = 1γ vitamin preparation. We tried to use dilutions in alcohol, but found that traces of alcohol were apt to increase irregularity of results.

We have assumed throughout that 1 curative pigeon dose = 1 vitamin B_1 International Unit [see Kinnersley, O'Brien and Peters, 1933].

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[1] An envelope opener is ideal for this purpose.

[2] Other apparatus for the estimation of oxygen uptake will of course do equally well.

[3] Erroneously described as 710 mm. in previous paper.

Note. Care should be taken to see that the tissue does not become permanently washed up and stuck upon the sides of the bottles. This happens especially with some avitaminous brains, for reasons which are not clear. It can usually be dislodged by careful manipulation.

Variation of catatorulin effect with concentration.

Fig. 1 shows a typical curve (*Exp. 316*) relating concentrations of crystalline vitamin B_1 to oxygen uptake for the 2nd hour. Within the hour period the general resemblance to an enzyme activity-substrate concentration curve is striking, if for concentration of substrate we substitute catatorulin concentration. For values from 0 to 0·1 vitamin B_1 unit, the uptake rises considerably; with higher concentrations, the increment is smaller. In the application to estimations, certain limitations appear. It is not practicable to obtain more than 16 samples from one brain, nor convenient to work with 2 brains. The experimental error is somewhat larger: though 2 samples give approximate values for rough tests, the highest accuracy can be reached only by an average of at least 4 samples. Therefore we require a means of reaching the probable shape of the curve from one or two points.

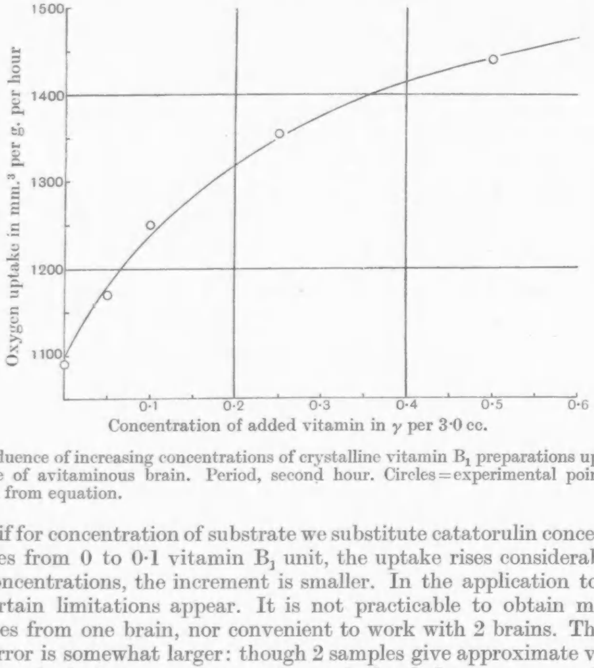

Fig. 1. Influence of increasing concentrations of crystalline vitamin B_1 preparations upon oxygen uptake of avitaminous brain. Period, second hour. Circles = experimental points. Curve drawn from equation.

A reasonable explanation for the shape of the curve in Fig. 1, is to assume that among other limiting factors the oxygen uptake depends upon the adsorption of catatorulin at some active centre in the brain tissue. This adsorption will depend upon concentration of the factor in the same way as adsorption of substrate upon an enzyme depends upon concentration of substrate. It is reasonable therefore to apply the Michaelis equation, assuming that lactate concentration may be regarded as maximum [see Gavrilescu *et al.*, 1932], and that concentration of catatorulin is the limiting factor.

Note. In this particular instance it seems that we must be dealing with a case of heterogeneous equilibrium, in which the particles of brain tissue may be regarded as practically stationary when compared with the vitamin B_1. This does not invalidate the use of the equation, because it can be also derived from Langmuir's conceptions of heterogeneous catalysis.

Theoretically, if

k_c = equilibrium constant,
v = velocity at concentration of added vitamin x (measured in mm.^{3}/g./hr.),
R = residual vitamin in tissue,
E = amount of enzyme (here directly proportional to tissue),

then $$v = \frac{k.E\,(x+R)}{k_c+(x+R)} \qquad \text{......(1),}$$

and if V = velocity when x is large, we have

$$k_c = (x+R)\left(\frac{V}{v}-1\right) \text{ for constant amount of enzyme system } E \quad \text{......(2).}$$

The tissue is chosen to be approximately 100 mg. The catatorulin concentration is measured in terms of γ or $10^{-2}\gamma/3$ cc. solution.

Now the mixed brain provides 12–16 samples of about 100 mg. each, making 4 quadruplicate estimations possible as a maximum. Two estimations are made with a standard catatorulin preparation at concentrations of $100 \times 10^{-2}\gamma$ (1γ) and $3 \times 10^{-2}\gamma$ respectively and one or two at arbitrary concentration. From these an average curve is drawn by calculating V from the relation

$$V = \frac{v}{x+R}\,(k_c + x + R) \qquad \text{......(3).}$$

It is found that the curves are best satisfied when $k_c = 10 \times 10^{-2}\gamma$, *i.e.* 1/20 vitamin B_1 unit gives 1/2 possible velocity, and when $R = 20 \times 10^{-2}\gamma$ for 100 mg. tissue. The latter is quite reasonable; it assumes a figure of 1 vitamin B_1 unit (pigeon dose) per g. of brain tissue as the limiting concentration possible for life.

The curve is then drawn, using for the points

$$v = \frac{x+R}{k_c+x+R}\,.\,V \qquad \text{......(4).}$$

That at $20 \times 10^{-2}\gamma$ will serve to test the equation. From the curve the concentration of the unknown can readily be found.

Correction for variation in the amount of the tissue. Variations are unavoidable in the amount of the tissue, though often they do not exceed ±15 mg. Change in the amount alters E and also R, R changing in direct relation with E. From equation (1) the effect of changes in tissue amount may be calculated.

If E_1 and E_2 = amounts of enzyme system, in mg. tissue per apparatus, and $(x + R_1)$ and $(x + R_2)$ are the amounts of vitamin (in $10^{-2}\gamma$) present in each bottle, R_1 and R_2 being the amounts of tissue vitamin corresponding to E_1 and E_2 respectively, it can be shown that

$$\frac{v_1}{v_2} = \frac{E_1}{E_2}\cdot\frac{\left(\frac{k_c}{x+R_2}+1\right)}{\left(\frac{k_c}{x+R_1}+1\right)} \qquad \text{......(5).}$$

In calculating results, E has always been taken into account because the figures are reduced to mm.^{3}/g./hr.; no account has been taken, however, of R in the past. Let us consider variations from the true value, introduced by this method of calculation in which R is considered as the amount in 100 mg. tissue. Table I gives results, calculated from equation (5) for $x = 0, 20, 100 \times 10^{-2}\gamma$; $k_c = 10 \times 10^{-2}\gamma$, $E_1 = 100$ mg. and E_2 varying.

From Table I it is seen that no allowance for R need be made with amounts of vitamin $100 \times 10^{-2}\gamma$ (1/2 B_1 unit); failure to consider R will make a distinct difference at $x = 20 \times 10^{-2}\gamma$, and more so when $x = 0$. This accords with

Table I.

C_2 (mg.)	R ($10^{-2}\gamma$)	x_1 $=0$	x_1 $=20\times10^{-2}\gamma$	x_1 $=100\times10^{-2}\gamma$
150	30	112·5	104	100·5
120	24	106	102	100
100	20	100	100	100
90	18	96·5	99·0	100
80	16	92·5	97·5	100
70	14	87·5	97·0	100
50	10	76	96·0	99

experience. With maximum amounts of vitamin it has often been noticed that the amount of tissue could be varied within wide limits without influencing the results. With estimations of residual uptake, or with small amounts of vitamin, unaccountable variations have often appeared. Reference to the notes of previous work in this laboratory shows that these may be largely eliminated by taking into account variation in R in these cases. The use of equation (5) and Table I seems to be thoroughly justified. Where necessary, therefore, results are now corrected in this manner.

EXPERIMENTAL RESULTS.

Exp. 1 gives a protocol of a typical experiment, with a discussion of its probable accuracy.

No. 414. 29. iii. 33. Avitaminous pigeon's brain (except cerebellum) was finely minced and mixed. It was distributed in 12 tared bottles of Barcroft apparatus, which had been previously

Readings.

Bottles No.	1		2		3		4	
Constant	3·01		2·78		2·94		3·05	
Tissue wt. (g.)	0·1072		0·0979		0·1220		0·1171	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Time 4.16 p.m.	2·5	—	2	—	2	—	2·5	—
5.16 ,,	38·0	35·5	38·5	36·5	43·5	41·5	45·5	43·0
6.16 ,,	70·0	32·0	70·5	32·0	77·5	34·0	82·5	37·0

Bottles No.	5		6		7		8	
Constant	2·78		2·76		2·73		2·98	
Tissue wt. (g.)	0·1177		0·1008		0·1099		0·0996	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Time 4.16 p.m.	0·5	—	2	—	4	—	3·5	—
5.16 ,,	45·5	45·0	42·5	40·5	52·0	48·5	40·5	37·0
6.16 ,,	84·5	39·0	79·0	36·5	95·0	43·0	73·5	33·0

Bottles No.	9		10		11		12	
Constant	3·04		2·81		3·25		3·48	
Tissue wt. (g.)	0·1119		0·1195		0·1073		0·0912	
	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)	(*a*)	(*b*)
Time 4.16 p.m.	3	—	4	—	2·5	—	3·0	—
5.16 ,,	42·5	39·5	51·0	47·0	36·0	33·5	32·0	29·0
6.16 ,,	76·5	34·0	90·5	39·5	66·5	30·5	58·0	26·0

Bottles No. 1, 2, 3, 4 contained 0·34γ 64·19 (crystalline vitamin B_1 preparation)
,, 5, 6, 7, 8 ,, 1·0γ 64·19 ,, ,,
,, 9, 10, 11, 12 ,, 0·5γ 78·27 ,, ,,

(*a*) Difference in readings in scale divisions.
(*b*) Scale divisions per hour of differential apparatus.

corked after filling with Ringer-phosphate lactate solution, volume 3·0 cc. *minus* 0·1–0·5 cc. to allow for addition of vitamin. The bottles were reweighed; the brain was subdivided with a glass stirrer; the freshly diluted and neutralised vitamin was immediately added; the bottles were twice evacuated *etc.* and placed in the bath at 38 ± 0·1°. Time from guillotining 40 minutes. Readings commenced at 52′ = 4.16 p.m.

Summary.

Vitamin		mm.^{3}/g./hr.		Average	
		First hour	Second hour	First	Second
$0 \cdot 34\gamma$	64·19	995	905	1040	900
		1040	915		
		995	815		
		1120	960		
1γ	64·19	1060	920	1120	990
		1110	995		
		1205	1060		
		1110	995		
$0 \cdot 5\gamma$	76·27	1070	930	1070	940
		1105	935		
		1010	920		
		1105	990		

The mean standard deviation for the 24 samples is 47·5 (48), and the standard error of mean $\frac{\sigma}{\sqrt{n}}$ for 4 observations is 24. Hence 2/3 of such tests should fall within ±24, and 21/22 within $\pm 2\epsilon = 48$. For 64·19, we have

	First hour	Second hour
$0 \cdot 34\gamma\ V =$	1230	1070
$1 \cdot 0\gamma\ V =$	1215	1070
Average	1220	1070

The standard error of mean (ϵ) for V will be $\frac{\sigma}{\sqrt{8}}$, *i.e.* $\frac{48}{2 \cdot 83} \times \frac{123}{110} = 19$ for first hour and $\frac{48}{2 \cdot 83} = 17$ for second hour.

Hence V for first hour $= 1220 \pm 38$ and for second hour 1070 ± 34, if we accept $\pm 2\epsilon$ as the limits between which 21/22 such tests must fall. We may remember that 2/3 of such tests would fall between the limits of 19 and 17 respectively, or approximately 1·6 %.

Fig. 2 shows the curves drawn through $V = 1220$ and 1070, with dotted curves for ±1·6 %.

From the curve, we learn that for 58·27, $0 \cdot 5\gamma = 0 \cdot 48\gamma$ and $0 \cdot 51\gamma$ respectively of 64·19. *Average* $0 \cdot 50\gamma$, *i.e.* the preparations are identical in catatorulin activity within experimental limits. On curve A (first hour), we have plotted the area representing $\pm\epsilon$. This is theoretically the area within which 2/3 of the tests should fall. Of course by increasing the number of observations to 2 in an experiment, and taking the mean average of 4 observations the standard error of an estimation is again reduced by half. The method should therefore reach with a few birds a much higher accuracy than does the curative or growth test for vitamin B_1.

Table II shows additional evidence of the concordance obtained between observed results and the points calculated from a theoretical curve with constant $k_c = 0 \cdot 1\gamma$. Considering the sources of error, including the difficulty of thoroughly mixing tissue, the observed results give a reasonable agreement with theory. Table III gives some of the comparisons which have been made already by the final methods upon crystalline specimens. Though preliminary, they show a satisfactory parallelism between catatorulin and vitamin B_1 effects in the preparations studied. If supported by further work, the method should prove an accurate means of assaying vitamin B_1. It is especially interesting to note that the catatorulin activity of the German preparation is approximately half that of our own, which is in striking agreement with the conclusions for vitamin B_1 activity by curative test.

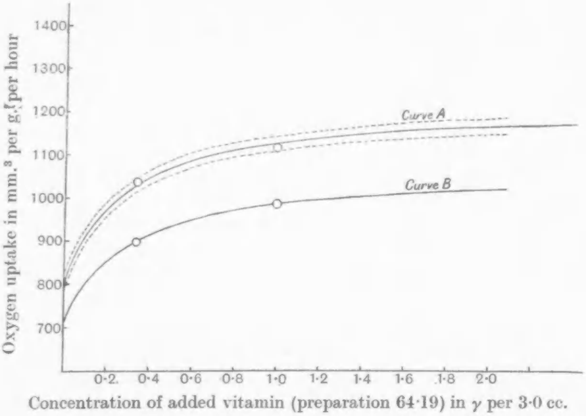

Fig. 2. Oxygen uptake and vitamin B_1 concentration. Curves A and B theoretical curves for first and second hour, drawn from the two experimental points (circles). Dotted curves represent $\pm\epsilon$ for curve A.

Table II. *Comparison of observed with calculated oxygen uptake in various experiments.*

Experiment	Amount of vitamin in $10^{-2}\gamma/3$ cc.	O_2 uptake in first hour: Observed	O_2 uptake in first hour: Theoretical	O_2 uptake in second hour: Observed	O_2 uptake in second hour: Theoretical
403	20	1315	1315	960	965
	10	1240	1235	910	905
404	10	1080	1090	700	685
	5	1050	1040	640	655
407	100	[1320]*	1530	940	945
	30	1380	1380	855	860
	15	1300	1290	800	800
408	100	1345	1400	960	970
	30	1250	1265	915	915
	15	1230	1180	810	815
410	100	1450	1450	1080	1090
	30	1380	1310	990	985
	15	1230	1225	860	915
411	100	1290	1295	985	970
	40	1215	1205	900	900
	20	1120	1120	840	840
414	100	1120	1125	990	990
	34	1040	1030	900	900
415	100	1280	1250	950	925
	34	1120	1140	810	840
417	100	1270	1290	940	965
	30	1195	1170	875	865

* This figure is a marked exception, and is possibly due to experimental error.

Table III.

A. *Catatorulin tests on vitamin B_1 preparations.*

Exp. No.	Unknown vitamin preparation	Standard used for comparison		Vitamin B_1 mm.^{3}		% diff.	Remarks
		53	64·19	First hour	Second hour		
380	63. A. 9	—	—	1790	1455	81·2	Triplicate
383	56·20 64·19	0·3 (56) = 0·37γ (1) = 0·37γ (2) 0·3 (64) = 0·27γ (1) = 0·2γ (2)	—	1720	1280	74·5	,,
403	Windaus *et al.*	—	0·2γW = 0·15γ (1) = 0·10γ (2) 0·4γW = 0·31γ (1) = 0·30γ (2)	1645	1205	73·5	,,
410	Windaus *et al.*	—	1·2γW = 0·75γ (1) = 0·6γ (2)	1570	1170	74·5	,,
414	78·27	—	0·5γ78 = 0·51γ (1) = 0·48γ (2)	1220	1070	88	Quadruplicate
415	78·27	—	0·4γ78 = 0·55γ (1) = 0·55γ (2)	1350	1000	74	Quintuplicate

(1) First hour; (2) Second hour.

B. *Amounts having similar activity.*

Preparation	Vitamin B_1 units	Catatorulin	B_1/C
64·19	10γ	10γ	1·0
German	17·8γ	16γ	1·1
78·27	11·1γ	8·5γ	1·3
56	11·2γ	6·0γ	1·85
53	10γ	6·3γ	1·6

DISCUSSION.

It is clear that the complicated nature of the systems studied precludes more than provisional conclusions. With the proviso that the catatorulin in vitamin B_1 crystals is not an actual substrate, it is interesting to compare k_c with k_m for the enzymes proper. Assuming a molecular weight of not less than 300 for factor, k_c is of the order of $10^{-8}M$. This is even a higher affinity than that found for the oxidising enzymes; it will be interesting if similar facts hold for k_c of other co-enzymes. So far as we know, this is the first measurement. It is reasonable and consistent with the facts to think that a co-factor would be more highly adsorbed than the substrate. In this case, of course, we are still uncertain of the nature of the substrate.

Variations in R (the residual vitamin) should theoretically influence the curve as shown in Fig. 3. If therefore R increases to $40 \times 10^{-2}\gamma$ per 100 mg. (2 pigeon doses/g.), we should expect to see only very small catatorulin effects with changes in added vitamin. This has been shown to be the case for the normal brain, and suggests that normal tissue contains at least 2 units vitamin B_1 per g. It is likely that with further experience estimations of R in small amounts of tissue will be possible by this method.

Theoretically there is no reason why these principles should not be used generally for the estimation of substrate with tissue preparations.

Exp. 1 shows that k_c does not change significantly during the 1st and 2nd hours, when working with crystalline vitamin preparations. This is found even though the total oxygen uptake decreases. It indicates that this decrease is due to a steady inactivation of the enzyme system, in which E progressively decreases.

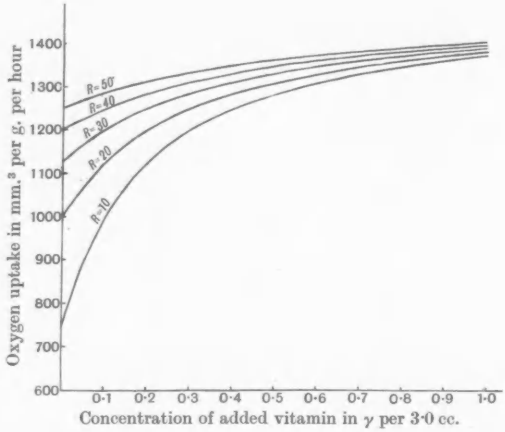

Fig. 3. Variation in position of theoretical curves for increasing concentrations of residual (tissue) vitamin (R); $V=1500$.

Summary.

1. A method is described for comparing the co-ferment effect upon the oxygen uptake of avitaminous birds' brain of two vitamin B_1 concentrates. The specific factor responsible is called "catatorulin."

2. Crystalline specimens of vitamin B_1 are active sources of catatorulin; distinct effects are shown with 0·1γ in 3·0 cc. solution.

3. There is a wide parallelism between vitamin B_1 and catatorulin activity, which suggests strongly that they are identical and that the method estimates vitamin B_1.

4. The affinity constant (k_c) for catatorulin is of the order of 10^{-8} M, which is greater than that of the oxidases for their substrates.

We are indebted to the Medical Research Council for grants in aid of this work, and also to Prof. Windaus and Dr Tschesche for the specimen of crystals used in Exps. 403 and 410.

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CXI. THE COMPONENT FATTY ACIDS AND GLYCERIDES OF THE MILK-FATS OF INDIAN GOATS AND SHEEP.

By DAS RAM DHINGRA.

From the Forman Christian College, Lahore, Panjab University.

(*Received May 1st, 1933.*)

The milk-fats from goats and sheep are used, although only to a small extent, as substitutes for those of cows and buffaloes in India. The component fatty acids and glycerides of these two fats have been studied by methods recently developed [Hilditch *et al.*, 1929; 1930; 1931] in the case of cow butter-fats, in order to compare their composition with those of the latter and, in certain respects, with beef and mutton tallows [Banks and Hilditch, 1931]. It may be noted, in passing, that "ghee" (the prepared butter-fat used in India on account of the difficulty of keeping ordinary butter fresh in the hot, humid climate) is defined in the Bombay "Adulteration of Ghee" Act as made from the butter-fat of either cows, buffaloes, goats or sheep.

The milk-fats used in the present investigation were specially prepared by churning the fresh milk and at once melting the resulting butter at 100°, the fats being separated by decanting and then filtering through a double layer of filter-paper to remove traces of water and caseinogen. The fats were stored *in vacuo* in sealed amber glass bottles.

The goat milk-fat was from the milk of animals at Lahore (Panjab) taken in January 1932; they had been fed on a winter diet of turnips, grass, zizyphus Jujuba tree leaves and fruits and barley meal. The yield of fat from different samples of the goat milk varied from 3·2 to 3·6 %. The sheep milk-fat was obtained in February 1932 from the milk of Leiah (Multan, Panjab) sheep, which had been fed on a winter ration of grass, zizyphus Jujuba leaves and fruits, turnips, carrots and barley meal; the milk contained from 4·0 to 4·2 % of fat.

The general characteristics of the milk-fats are given in Table I.

Table I.

	Goat milk-fat.	Sheep milk-fat.
Colour	Almost white (cream tinge)	Pale yellow
Sap. equiv.	238·1	232·4
Iodine value	28·8	32·1
Acid value	2·9	2·2
Reichert-Meissl value	31·7	33·9
Polenske value	8·2	8·0
Kirschner value	19·9	22·8
Setting-point (fat)	23·7°	22·2°
Valenta number	21·5°	21·6°
Refractive index $n_D^{40°}$	1·4523	1·4526

Some values recently reported for these fats by other investigators are collected in Table II:

Table II.

	Saponification equivalent	Iodine value	Reichert-Meissl value	Polenske value	Kirschner value
(i) Goat butter-fat:					
Egyptian [Triman, 1913]	—	—	20·8–22·9	4·9–6·5	—
English [Knowles and Urquhart, 1924]	—	24·7–36·9	24·5–27·8	4·9–8·7	16·8–19·0
[Bardisian, 1926]	241·3	28·1	—	—	—
Egyptian [Atkinson, 1928]	228–238	32·5–41·2	20–28	4·0–8·7	—
(ii) Sheep butter-fat:					
[Bardisian, 1926]	241·3	32·3	—	—	—
[Faltin and Dedinzky, 1930]	—	—	22·5–39·4	2·2–6·9	15·8–21·0

The Polenske values of these fats are thus decidedly higher than those of cow butter-fats, the Kirschner values are somewhat low in relation to the Reichert-Meissl values as compared with cow butter-fats, and the saponification equivalents also tend to be lower than those of cow butter-fats.

Component fatty acids of goat and sheep milk-fats.

The component fatty acids were quantitatively determined in each case by the method described fully by Hilditch and Jones [1929] and Hilditch and Sleightholme [1930], the mixed acids from 350 g. of fat being employed in each case. The final results for the two fats are summarised in Table IV; but the details of the fractionation data for the goat butter-fatty acids are first quoted in Table III, since comparison of these with the similar details given for cow butter-acids by the workers mentioned [1929, pp. 80–82; 1930, pp. 1102, 1103] helps to indicate the nature of the differences between the respective fats.

Oxidation of the dilute aqueous solution of the potassium salts of the acids from the penultimate fraction of the "liquid" esters from the sheep milk-fat led to the identification of a dihydroxystearic acid (M.P. 130°) and a tetrahydroxystearic acid (M.P. 174°), thus showing that the ordinary $\Delta^{9:10}$-oleic and $\Delta^{9:10,\ 12:13}$-linoleic acids were the unsaturated components of the fat. Arachidic acid (M.P. 74·5°) was definitely characterised in the hydrolysed residual fraction of the sheep milk-fat "solid" esters, and its presence was also indicated (although it was not obtained in the pure condition) in the corresponding fraction of the goat milk-fat esters.

An early fractionation analysis of the sheep milk-fat acids by Crowther and Hynd [1917] does not agree with the present data; for example, these authors give the respective percentages (wt.) of butyric, palmitic and oleic acids as 6·5–6·6, 12·8–13·9 and 40·6–40·7. The differences are doubtless accounted for by the facts that they fractionally distilled the esters of the whole of the mixed acids and so did not obtain a sufficient degree of separation, and that they did not allow for the presence of linoleic acid in the unsaturated acids. The limitations involved in Crowther and Hynd's fractionation procedure have already been discussed elsewhere in the case of cow butter-fats [Channon, Drummond and Golding, 1924; Hilditch and Jones, 1929].

The main differences between the component fatty acids of the goat and sheep butters now under discussion and those of cow butter-fats as given by Hilditch *et al.* [1929; 1930; 1931] are the much higher content of caprylic and capric acids (and, to a less extent, of caproic acid), and the lower percentages

Table III. *Fatty acids of goat milk-fat.*

(*a*) *Acids volatile in steam.*

No.	g.	B.P. (° C.)	Pressure	Mean equivalent	Acids: Butyric	Caproic	Caprylic	Capric	Lauric	Oleic	Non Sap.
1	In aqueous solution (5100 cc.)	—	—	—	2·06	—	—	—	—	—	—
2	In recovered ether (2220 cc.)	—	—	—	0·12	—	—	—	—	—	—
3	20·28	35–120	Atmospheric	—	1·97	—	—	—	—	—	—
4	4·35	120–160	„	—	2·81	—	—	—	—	—	—
5	1·62	160–165	„	—	1·59	—	—	—	—	—	—
6	2·67	165–178	„	101·5	1·22	1·45	—	—	—	—	—
7	2·02	120–128	Reduced	113·6	0·14	1·88	—	—	—	—	—
8	1·99	128–132	„	117·6	—	1·85	0·14	—	—	—	—
9	2·56	135–145	„	126·0	—	1·53	1·03	—	—	—	—
10	0·92	146–147	„	130·8	—	0·39	0·53	—	—	—	—
11	2·17	147–153	„	135·9	—	0·54	1·63	—	—	—	—
12	2·66	190–194	„	146·9	—	—	2·34	0·32	—	—	—
13	2·41	205–223	(Iodine Value 3·5)	165·9	—	—	0·65	1·67	—	0·09	—
14	2·39	Residue	(Iodine Value 15·2)	208·0	—	—	—	1·22	0·64	0·34	0·19
					9·91	7·64	6·32	3·21	0·64	0·43	0·19

(*b*) *Acids non-volatile in steam, 302 g.*

(Sap. equiv. 247·4; Iodine Value 31·0.)

	Lead salt separation: g.	%	Corresponding esters: S.E.	Iodine Value
"Solid" acids S	94·7	46·3	274·3	5·0
"Liquid" acids L	110·0	53·7	250·3	53·5

(*c*) *Fractionation of methyl esters.*

(i) Esters of "solid" acids S.

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 1	30·94	122–142°	262·0	2·3
S 2	8·77	144–146	272·3	3·2
S 3	5·80	145–148	275·2	4·7
S 4	4·46	148–151	279·2	5·3
S 5	4·46	151–154	283·2	7·0
S 6	6·86	155–161	291·6	8·6
S 7	6·87	Residue	298·6	13·9

Refractionations

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
S 11	2·53	125–150°	252·4	2·0
S 12	4·55	150–156	257·9	1·5
S 13	4·50	155–159	257·7	1·0
S 14	3·14	156–160	256·3	1·5
S 15	4·67	159–163	266·2	2·1
S 16	3·77	Residue	270·5	3·9

(ii) Esters of "liquid" acids L.

Primary fractions

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 1	25·60	70–135°	200·4	9·5
L 2	5·61	136–149	266·3	47·5
L 3	5·46	149–160	279·0	66·3
L 4	7·68	161–162	291·8	83·5
L 5	5·99	163–169	293·4	86·0
L 6	5·91	169–171	295·9	90·7
L 7	6·00	171–175	295·4	89·4
L 8	8·19	Residue	319·3	76·9

Refractionations

No.	g.	B.P./1 mm.	Sap. eq.	I.V.
L 11	2·40	50– 76°	171·2	2·5
L 12	4·47	76– 78	182·0	4·0
L 13	3·05	78– 84	188·0	3·5
L 14	3·05	85–102	202·5	4·3
L 15	3·09	103–115	222·4	7·0
L 16	3·84	Residue	249·6	28·3

Table IV. *Summarised experimental data for fatty acids of goat and sheep milk-fats.*

		Acids non-volatile in steam		Component fatty acids		
				Including unsaponifiable	Excluding unsaponifiable	
Acid	Volatile acids	Solid acids S	Liquid acids L	% (wt.)	% (wt.)	% (mols.)
		Goat milk-fat.				
	8·6 %	42·3 %	49·1 %			
Butyric	3·01	—	—	3·01	3·0	7·6
Caproic	2·33	—	—	2·33	2·3	4·5
Caprylic	1·93	—	2·04	3·97	3·9	6·2
Capric	0·98	—	7·53	8·51	8·6	11·1
Lauric	0·20	—	4·34	4·54	4·6	5·1
Myristic	—	6·24	5·16	11·40	11·5	11·2
Palmitic	—	24·32	0·23	24·55	24·7	21·5
Stearic	—	9·24	—	9·24	9·3	7·3
Arachidic	—	0·14	—	0·14	0·1	0·1
Oleic	0·14	2·36	27·86	30·36	30·5	24·2
Linoleic	—	—	1·51	1·51	1·5	1·2
Unsaponifiable	0·01	—	0·43	0·44	—	—
		Sheep milk-fat.				
	9·14 %	37·61 %	53·25 %			
Butyric	3·32	—	—	3·32	3·3	8·4
Caproic	2·80	—	—	2·80	2·8	5·4
Caprylic	1·55	—	2·21	3·76	3·8	5·8
Capric	1·18	—	6·63	7·81	7·8	10·1
Lauric	0·15	—	5·21	5·36	5·4	6·0
Myristic	—	6·53	5·56	12·09	12·2	11·8
Palmitic	—	19·45	3·97	23·42	23·5	20·4
Stearic	—	6·33	0·55	6·88	6·9	5·4
Arachidic	—	1·85	—	1·85	1·9	1·3
Oleic	0·14	3·45	24·60	28·19	28·3	22·2
Linoleic	—	—	4·07	4·07	4·1	3·2
Unsaponifiable	—	—	0·45	0·45	—	—

of palmitic and oleic acids in the former as compared with the latter. This is, of course, in accordance with the characteristic features of the Polenske, Kirschner and Reichert-Meissl values (especially the high Polenske values) of the goat and sheep milk-fats, to which attention has already been directed. The apparently close similarity between the component acids of these two classes of milk-fats, and their differences from the cow milk-fat acids, will be clearly seen from the comparison of the molar percentages of the component acids which follows in Table V. For reasons given by Hilditch and Sleightholme [1930; 1931], the relative number of molecules of each acid present, rather than the relative masses of the acids, forms the best basis for comparison; while additional information may be gained from consideration of the combined amounts of the four groups of acids, butyric-lauric, myristic-palmitic, stearic(-arachidic), and oleic-linoleic, which are found in the various milk-fats.

The combined data in Table V show the marked increase in the butyric-lauric acid group, apparently mainly at the expense of the unsaturated (oleic) acids; but further inspection reveals that the palmitic acid content is also definitely lower than in the majority of cow butter-fats. In each 100 molecules of mixed fatty acids, there are about 11–12 molecules more of caprylic + capric acids in the goat and sheep milk-fats than in those of the cows. Correspondingly, in an English pasture-fed cow butter of normal oleic acid content, there are

Table V. *Comparison of the molar distribution of the component acids of goat, sheep and cow milk-fats.*

Fat	Goat butter	Sheep butter	Cow butters [Hilditch and Sleightholme, 1931]		
			Autumn fed (English)	Early summer pasture (English)	Pasture-fed December (New Zealand)
Butyric	7·6	8·4	8·4	8·9	9·2
Caproic	4·5	5·4	3·5	2·7	3·4
Caprylic	6·2	5·8	2·7	2·0	2·2
Capric	11·1	10·1	2·9	3·0	4·2
Lauric	5·1	6·0	4·1	4·7	4·7
Myristic	11·2	11·8	7·2	10·9	11·5
Palmitic	21·5	20·4	27·1	24·3	25·0
Stearic	7·3	5·4	6·4	5·4	9·5
Arachidic	0·1	1·3	0·7	—	0·5
Oleic	24·2	22·2	33·9	34·6	26·1
Linoleic	1·2	3·2	3·1	3·5	3·7
Butyric-lauric	34·5	35·7	21·6	21·3	23·7
Myristic-palmitic	32·7	32·2	34·3	35·2	36·5
Stearic(-arachidic)	7·4	6·7	7·1	5·4	10·0
Oleic-linoleic	25·4	25·4	37·0	38·1	29·8

about 10–12 mols. % more of this acid than in the Indian goat and sheep butters; while, in a more saturated cow butter-fat (the New Zealand sample in Table V), there are about 7 mols. % more of oleic + stearic acid than in the latter. The chief difference, taking the component acids of the fats as a whole, therefore seems to reside in the varying proportions of the caprylic-capric and oleic(-stearic) acid groups, with a subordinate corresponding variation in the content of palmitic acid. Further information on these differences emerges, however, from the study of the glyceride structure of the goat and sheep milk-fats which is recorded later in this paper.

It may be pointed out here that the two fats under discussion were from goats and sheep which were respectively reared in different localities of the Panjab, about 300 miles apart. The diet of both the goats and the sheep was, however, almost the same, whilst the milk was taken at the same season (January-February, 1932) when the temperature range in each place was almost identical (minimum 35°–maximum 70° F.). In the case of cow butter-fats, Hilditch and Sleightholme were able, to some extent, to correlate a fall in oleic acid content with the change from outdoor (summer) to indoor (winter) conditions or corresponding changes in diet, and with seasonal changes in temperature. The same factors might be considered to be operative in the present instances, but against this may be set the facts that the Indian winter temperature is more equivalent to that of spring or early summer in a temperate climate, and that the notable content of caprylic and capric acids is a feature clearly connected with the species of animal. The small content of these acids in the cow milk-fats has not been observed to increase in consequence of any alteration in temperature or feeding, *etc.*, conditions so far studied.

Component glycerides of the goat and sheep milk-fats.

The glyceride structure of the two milk-fats was studied by determining the proportion of fully-saturated glycerides present and the component fatty acids present in the latter, as described by Hilditch and Jones [1929]; information as to the components of the mixed saturated-unsaturated glycerides present was deduced from the combined data for the component acids of the whole fat and of the fully-saturated glycerides. The details of the fully-saturated glycerides obtained by the oxidation of acetone solutions of goat and sheep milk-fats with potassium permanganate are given in Table VI.

Table VI. *Determination of fully-saturated glyceride (F.S.G.) content.*

(i) Analytical data.

			Purification of crude fully-saturated glycerides							
				"A"		"B"		Acidic products		
Milk-fat	Weight taken g.	Crude F.S.G. g.	Weight taken g.	g.	Acid No.	g.	Acid No.	g.	Acid No.	F.S.G. % (weight)
Goat	300·0	115·4	114·3	103·45	Nil	4·67	22·0	5·18	91·6	36·3
Sheep	300·0	109·5	108·8	97·10	Nil	4·07	27·2	7·03	85·8	33·7

(ii) Summary of observed values.

		Acids in whole fat		Fully-saturated glycerides		
Milk-fat	Iodine value	Saturated % (mol.)	Unsaturated % (mol.)	Weight %	Mols. %	"Association ratio" in non-fully-saturated part. Mols. saturated acid per mol. unsaturated acid
Goat	28·8	74·6	25·4	36·3	39·3	1·40
Sheep	32·1	74·6	25·4	33·7	36·8	1·49

The results of determinations of the component fatty acids in these fully-saturated glycerides are given in Table VII.

Table VII.

				% (weight)	% (mol.)
Acid	Volatile acids %	Acids non-volatile in steam %	Total %	(excluding unsaponifiable matter)	
	Fully-saturated glycerides of goat milk-fat.				
	14·4 %	85·6 %			
Butyric	3·94	—	3·94	4·0	9·4
Caproic	4·02	—	4·02	4·1	7·2
Caprylic	3·40	0·62	4·02	4·1	5·8
Capric	3·04	8·60	11·64	11·8	14·2
Lauric	—	7·83	7·83	7·9	8·2
Myristic	—	13·86	13·86	14·0	12·7
Palmitic	—	38·65	38·65	39·1	31·6
Stearic	—	14·63	14·63	14·8	10·7
Arachidic	—	0·27	0·27	0·2	0·2
Unsaponifiable	—	1·14	1·14	—	—
	Fully-saturated glycerides of sheep milk-fat.				
	16·05 %	83·95 %			
Butyric	4·23	—	4·23	4·2	9·8
Caproic	4·68	—	4·68	4·7	8·2
Caprylic	3·80	—	3·80	3·8	5·4
Capric	2·28	6·80	9·08	9·1	10·8
Lauric	1·06	12·72	13·78	13·8	14·1
Myristic	—	17·80	17·80	17·9	15·9
Palmitic	—	33·82	33·82	34·0	26·9
Stearic	—	12·43	12·43	12·5	8·9
Unsaponifiable	—	0·38	0·38	—	—

The molar "association ratios" in the final column of Table VI permit limiting values to be calculated for the possible proportions of either mono-unsaturated di-saturated and di-unsaturated mono-saturated glycerides, or mono-unsaturated di-saturated and tri-unsaturated glycerides which may be present. It is probable that the proportion of triolein in milk-fats is extremely small [*cf.* Amberger, 1918], and that the minimum limits for mono-unsaturated derivatives and the maxima for the di-unsaturated class given in Table VIII are not far removed from the proportions actually present.

Table VIII. *Limiting values for the molar percentages of the four types of mixed glycerides in the goat and sheep milk-fats.*

			Glycerides (mols. %)			
Milk-fat	Iodine value	"Association ratio"	Fully-saturated	Mono-unsaturated di-saturated	Di-unsaturated mono-saturated	Tri-unsaturated
Goat	28·8	1·40	39·3	45·5–53·1	15·2–0	0–7·6
Sheep	32·1	1·49	36·8	50·2–56·7	13·0–0	0–6·5

As in cow butter-fats, the proportion of fully-saturated components of goat and sheep milk-fats is clearly a function of the relative amounts of saturated and unsaturated acids in the whole fats; also, the content of fully-saturated glycerides approaches to some extent that of the latter (i) in synthetic glycerides of the same degree of unsaturation [Bhattacharya and Hilditch, 1930] and (ii) calculated on the assumption that the amount of fully-saturated glycerides is proportional to the cube of the content of the saturated acids in the whole fats. At the same time, as Table IX illustrates, the amount of fully-saturated glycerides in each of the present cases is somewhat less, for a given ratio of saturated to unsaturated acids in the whole fat, than that characteristic of cow milk-fats (of which two specimens of somewhat similar iodine value are quoted. It will be noticed, also, that in the goat and sheep fats there is a considerably higher ratio of saturated to unsaturated acids in the mixed saturated-unsaturated glycerides than in the cow butter-fats.

Table IX.

			Fully-saturated glycerides % (mols.)			
	Iodine value	Total saturated acids % (mols.)	Observed	Calculated $y \propto x^3$	From synthetic glycerides curve	Association ratio
Goat butter	28·8	74·6	39·3	41·7	37·5	1·40
Sheep butter	32·1	74·6	36·8	41·7	37·5	1·49
*Cow butter II	31·6	72·4	41·3	38·0	34·0	1·11
*Cow butter V	34·5	70·2	39·6	34·5	30·5	1·07

* [Hilditch and Sleightholme, 1930.]

Closer examination of the component fatty acids present in the fully-saturated and non-fully-saturated parts of the fats reveals some interesting features. For example, Hilditch and Sleightholme [1931] observed remarkable constancy in the component acids of fully-saturated glycerides from six pasture-fed cow butter-fats which differed widely in iodine value and fully-saturated glyceride content, but the corresponding components of the goat and sheep butter-fats are by no means the same (*cf.* Table VII). In particular the fatty acids of the fully-saturated components of the goat and sheep fats include respectively molar contents of 31·6 and 26·9 % of palmitic acid, in contrast to the values 39·3–43·1 % obtained by the former authors for cow butters; whilst the stearic acid values (10·7 and 8·9 %) contrast equally with the almost constant figure (14·2–15·8 %) for the six cow butter-fats. The main differences between the two classes of milk-fat fully-saturated glycerides are thus the much higher proportion of the butyric-lauric acid group, and the correspondingly lower proportions of palmitic and stearic acids in the cases of the goat or sheep as compared with that of the cow.

These differences are illustrated in Table X, which gives a comparison of the proportions of the butyric-lauric acid group, and of myristic, palmitic, stearic (with arachidic) and oleic (with linoleic) acids in the whole fats, and their fully-saturated and non-fully-saturated components. As in Table V, the data given by Hilditch and Sleightholme for three pasture-fed cow butter-fats are added for comparison with the goat and sheep butter-fats.

Table X. *Molar distribution of fatty acids in goat, sheep and cow butter-fats.*

				Cow butters [Hilditch and Sleightholme, 1931]		
Section	Acids	Goat butter	Sheep butter	Autumn fed (English)	Early summer pasture (English)	Pasture-fed December (New Zealand)
Whole fat	Butyric-lauric	34·5	35·7	21·6	21·3	23·7
	Myristic	11·2	11·8	7·2	10·9	11·5
	Palmitic	21·5	20·4	27·1	24·3	25·0
	Stearic(-arachidic)	7·4	6·7	7·1	5·4	10·0
	Oleic-linoleic	25·4	25·4	37·0	38·1	29·8
Fully-saturated	Butyric-lauric	44·8	48·3	28·6	30·5	29·6
	Myristic	12·7	15·9	13·2	15·1	14·9
	Palmitic	31·6	26·9	43·1	39·5	39·9
	Stearic(-arachidic)	10·9	8·9	15·1	14·9	15·6
Saturated-unsaturated	Butyric-lauric	27·7	28·4	19·0	18·3	19·8
	Myristic	10·4	9·4	5·0	9·4	9·2
	Palmitic	15·1	16·5	20·9	18·7	15·0
	Stearic(-arachidic)	5·1	5·5	4·1	2·2	6·6
	Oleic-linoleic	41·7	40·2	51·0	51·4	49·4

Especial interest attaches to the figures deduced for the component acids of the mixed saturated-unsaturated glycerides, for here the contents of myristic, palmitic and stearic acids are very similar in all three types of milk-fat—cow, sheep and goat; the concordance is, indeed, almost exact between the cow fat in the final column (which was of the same order of total unsaturation (iodine value) as the goat and sheep fats) and the latter. In these mixed component glycerides, therefore, in all three fats, the only essential difference is that, in 100 molecules of fatty acids, there are about 10 molecules less of unsaturated C_{18} acids, and about 10 molecules more of the butyric-lauric group, in the goat and sheep fats as compared with cow butter-fats. Moreover, the relative increase in the butyric-lauric group of acids is most marked in that of capric and caprylic acids. These features seem to offer considerable support to Hilditch and Sleightholme's suggestion that the lower fatty acids in milk-fats are formed at the expense of C_{18} acids, *i.e.* a precursor of stearic or oleic acid in a tallow may, in the course of milk-fat metabolism, appear instead as a lower fatty acid, with the proviso that only one molecule of any of the lower acids is produced in milk-fat for each molecule of stearic (oleic) acid which otherwise would have resulted in a body-fat.

Finally, it may be pointed out that the relationships between sheep milk-fat and sheep tallows appear to be quite parallel with those disclosed between cow butter-fats and beef tallows [Banks and Hilditch, 1931]; the contents of myristic and palmitic acids in the whole fats and in their non-fully-saturated components, are closely similar, although (as in the analogous comparison between sheep and cow milk-fats) this accordance is lacking in the fully-saturated portions of sheep, butter and mutton tallow.

SUMMARY.

The component fatty acids of Indian goat and sheep milk-fats, and of their fully-saturated glycerides, have been determined in detail, and from these data the general structure of the glycerides has been deduced. The results have been compared with those previously obtained by other workers for cow butters and animal body fats.

The fatty acids of the milk-fats of goats and sheep, from two different places in the Panjab, but fed upon almost similar rations in the same winter season, are closely similar in composition, but differ from those of cow or buffalo butters in the higher content of the lower fatty acids (capric, caprylic and caproic) and the lower content of C_{18} unsaturated acids in the former than in the latter. The two sets of fats can be readily distinguished from each other by the Polenske value.

The content of fully-saturated glycerides in the two fats seems to be a function of the mean unsaturation, as in cow milk-fats. The component fatty acids of the fully-saturated glycerides are not so closely alike as in those of cow butter-fats, and also differ from the latter. In the mixed saturated-unsaturated glycerides, however, the molar contents of myristic, palmitic and stearic acids are very similar to those of the corresponding portions of cow butter-fats, and the only difference is that, in the goat and sheep milk-fats, there is about 10 % (mols.) less of C_{18} unsaturated acids and 10 % (mols.) more of the butyric-lauric acid group (the increase being mainly in capric and caprylic acids). The excess of capric and caprylic acids, as compared with cow butter-fats, therefore appears to be almost wholly at the expense of oleic acid.

The general properties and component glycerides of these two milk-fats are not far removed from those of the cow or buffalo, and there is, from this point of view, no reason why they should not be used as substitutes for the latter.

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CXII. EXPERIMENTS ON THE ORIGIN AND FUNCTION OF BERBERINE IN *BERBERIS DARWINII.*

By BENJAMIN THORNTON CROMWELL.

From the Department of Biochemistry, University of Liverpool.

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The classical researches of Pictet [1905; 1907] led him to conclude that alkaloids were waste products of the metabolic activity of the plant, analogous to urea, ethereal sulphates *etc.* of the animal body, and he supposed that they were produced in two stages (*a*) by the resolution of nitrogenous bodies such as proteins or chlorophyll into more simple basic substances, and (*b*) subsequent condensation of these simple bodies with the active products of metabolism, *e.g.* aldehydes, with the formation of the alkaloid nucleus. Research on these lines in recent years has been sporadic, and the results are difficult to correlate because of diversity in the nature and origin of the alkaloids studied. Thus Annett [1920] studied the effect of sodium nitrate on the production of latex by *Papaver somniferum* and found a large increase with but little variation in its morphine content. Mothes [1928] showed that the concentration of nicotine in the leaves of *Nicotiana* increased as the leaf developed, while Koenig [1931] found an increased production of alkaloid if nitrogenous fertilisers were applied. Ripert [1921] studied the effect of etiolation on the atropine and hyoscyamine content of young shoots of *Atropa* and found a considerable increase in both leaves and stalks. In addition, Sabalitschka [1923] and Sabalitschka and Jungermann [1925] observed no decrease in the alkaloid content of the seeds of *Lupinus* and *Strychnos* on germination. All these results tend to confirm Pictet's assertion that alkaloids are waste products, but throw very little extra light on the question of a possible connection between alkaloid production and protein metabolism.

In the present research an attempt has been made to unify some of these earlier findings by studying the metabolism of berberine in *Berberis Darwinii*. The experiments have been continued throughout two seasons, and have been planned to throw light on (1) the variation in berberine content with age, (2) the effect of applying nitrogenous fertilisers and of the withdrawal of essential elements, (3) the changes brought about by etiolation, (4) disturbances in the normal distribution of berberine brought about by ringing of the bark. The results obtained establish in certain cases an undoubted connection between protein breakdown and berberine synthesis.

EXPERIMENTAL.

Materials used. The plants were obtained as required from nurseries and the ages are calculated from the time of the first transplantation. The various plant members were cut into small pieces and dried at 105–110° for one day, finely powdered in a coffee mill and dried for the further period of one day.

Estimation of berberine. 4 to 5 g. of the powdered material were extracted with alcohol in a Soxhlet apparatus until the extract was no longer coloured yellow, and the berberine was estimated in the alcoholic extract by the method of Richter [1914] which is based upon the fact that berberine in one of its isomeric forms is soluble in ether. The normal and crystalline form (ammonium form) is soluble in water and insoluble in ether; by the action of alkali this is converted into the presumed "aldehyde form" which is soluble in ether and almost insoluble in water. The procedure was as follows.

The alcoholic extract was evaporated to dryness on a water-bath and the residue taken up with hot water, which precipitates resinous matter. The resin was filtered off and the aqueous solution evaporated to a small bulk (20 cc.). 10 cc. of 15 % NaOH solution were added and the alkaline solution was shaken with 110 cc. of ether for 10–15 minutes, 5 cc. of a 2 % solution of gum tragacanth being added prior to shaking. 55 cc., representing one half of the original volume of ether added, were removed for the subsequent estimation. The ethereal extract was then treated with a saturated solution of picrolonic acid in ether until precipitation was complete. The precipitate of alkaloid picrolonate was allowed to stand overnight and filtered through a Gooch crucible. The precipitate was washed with ether to remove picrolonic acid, dried at 105° and weighed. The weight multiplied by the factor 0·561 gives the equivalent amount of berberine. The accuracy of the method was tested by applying it to a commercial sample of berberine hydrochloride. A recovery of 100 ± 5 % was obtained.

PART I.

The seasonal variation in the berberine content of roots and stems.

The first investigation was to determine whether the berberine content of the different tissues varied during the season otherwise than as a result of increased age. For this purpose a large number of 3- and 5-year-old plants were investigated periodically from October until the following August. It is to be noted that each series of estimations was carried out on one plant, and that the successive results have all been obtained on different plants, the age of which is believed to have been 3 or 5 years. These results are summarised in Table I.

Table I. *Mean values showing the seasonal variation in berberine content (g./100 g. dry tissue).*

	3-year-old plants			5-year-old plants		
Date	Roots (whole)	Main stem	Subsidiary branches	Roots (whole)	Main stem	Subsidiary branches
Oct.–Dec.	1·24	0·24	0·10	1·41	0·38	0·17
Jan.–Mar.	1·21	0·21	0·10	1·36	0·38	0·12
April–May	1·25	0·28	0·12	1·39	0·39	0·14
June–Aug.	1·34	0·36	0·13	1·57	0·46	0·17

From the figures in Table I it will be seen that the highest concentration of berberine occurs in the roots: the main stems contain about one-fifth of the quantity in the roots, and the subsidiary branches about one-tenth. Furthermore, there is little change in the berberine content of the tissues during the period October to March. From March onwards the figures rise to a maximum at the end of the summer.

It will be seen that the berberine accumulates in the tissues during the period of active photosynthesis, and the enhanced level is maintained until the following spring, for the 5-year plants contain more berberine in all their tissues than the 3-year plants.

Further, figures for the distribution of alkaloid in 7-year-old plants were obtained from plants used for cultural experiments. In these experiments the initial concentrations, before the application of the various nutrient solutions to the plant, were determined by removing from each plant samples of subsidiary roots, main stems and subsidiary branches. In addition, one plant, age 30 years, was also investigated as follows in January 1931: height of plant 7 ft. 6 in.: secondary branch analysed 5 ft. 6 in. above ground: main stem analysed at levels 5 ft., 4 ft., 3 ft., 1 ft. 6 in. above ground: sections cut at 1 ft. 6 in. above ground and of main root 3 in. below soil surface. These results are set out in Tables II and III, while Table IV records the average figures for the berberine content of 3, 5, 7 and 30-year-old plants.

Table II. *Concentration of berberine in tissues of 7-year-old plants (g./100 g. of dry tissue).*

Number	Subsidiary root	Main stem	Subsidiary branches
3	2·14	0·53	0·06
5	1·22	0·36	0·08
7	2·06	0·43	0·14
8	1·57	0·64	0·13
9	1·88	0·91	0·20
10	1·94	0·43	0·11
11	1·58	0·48	0·12
12	1·86	0·40	0·09
13	1·83	0·47	0·11
14	2·45	0·37	0·07
15	1·96	0·47	0·09
16	1·65	0·41	0·11
17	1·81	0·40	0·13
Average	1·84	0·48	0·11

Young active growing shoots. Mean value 0·04 %.
Young parenchymatous roots. Mean value 1·41 %.

Table III. *Distribution of berberine in tissues of a 30-year-old plant (g./100 g. of dry tissue).*

	Bark	Wood
Tertiary branch	—	0·33
Secondary branch	—	0·76
Main stem apex	2·67	—
	2·75	0·76
	3·58	0·85
Soil level	4·93	1·53
Root (surface)	11·17	2·86
(1 foot below surface)	10·94	2·60
(secondary branches 6 inches below surface)	—	2·80

Table IV. *Average concentration of berberine in various tissues of 3, 5, 7 and 30-year-old plants.*

Age (years)	Roots (whole)	Roots (subsidiary)	Main stem	Branches (subsidiary)
3	1·27	—	0·29	0·12
5	1·44	—	0·40	0·15
7	—	1·84	0·48	0·11
30	bark 11·0	—	2·67–4·93	0·33–0·76
	wood 2·80	2·80		

All these figures are consistent in showing the progressive increase in the berberine content with increase in age.

In no plant was sufficient berberine present in the leaves to enable a chemical estimation by this method to be carried out. Attempts were therefore made to detect it by means of the colour reaction with chlorine water and hydrochloric acid, which varies from pink to red according to the amount of alkaloid present. In none of the young leaves except in those of the 30-year-old plant could berberine be detected by this means. On the other hand it was found in the old leaves of all plants irrespective of age.

Histological distribution of berberine in the various tissues.

Very young roots (parenchymatous) triarch root bundles. Alkaloid is most abundant in a ring of parenchymatous tissue surrounding the vascular bundle. Where phellogen is developed this forms the distal boundary of the berberine-containing tissue. The walls of the xylem elements where developed are impregnated with the alkaloid, but the phloem is free.

Fibrous roots. Further penetration of the xylem (which has developed to a considerable extent) has taken place. Ring of tissue surrounding the vascular bundles still the richest in berberine, but many cells of the cortex show the presence of alkaloid. The phloem is free.

Main and subsidiary roots. Alkaloid is distributed throughout the section of the root with the exception that the phloem remains free.

Main stem. The distribution is similar to that of the root but the upper sections show a fall in concentration more especially in the bundle sheaths and (where present) pith. The phloem is free.

Young shoots. These show the presence of chlorophyll corpuscles in the ring internal to the phellogen—no berberine could be detected in the bundle sheaths or vessels.

Etiolated shoots. These appear to have a greater concentration of berberine at the base than at the apex and are richer than the woody stems from which they spring. In section, the ring of tissue (inner-cortex) immediately surrounding the vascular bundles and interior to the phellogen is engorged, the wood fibres show definite impregnation, while the vessels and phloem are quite free.

Discussion of results.

Examination of the distribution of alkaloid throughout the various organs and tissues reveals the fact that berberine occurs in largest amounts in (*a*) organs of the plant which normally grow in absence of light, *i.e.* roots and rhizomes, (*b*) cortical tissues, the older and external cells of which are constantly being cast (in this connection it is worthy of note that *Berberis* possesses a deep-seated phellogen and consequently a large cortical area), (*c*) tissues which are known chiefly to perform mechanical functions, *e.g.* sclerenchymatous bundle sheaths and xylem. These observations lead to the deduction that in plants grown in normal conditions of environment the alkaloid is deposited in tissues which take no definite part in metabolic activities and which are made up principally of dead cellular elements. In etiolated shoots however, the highest concentration of berberine occurs in the ring of living parenchymatous cells surrounding the vascular bundles. It appears that this ring of cells surrounding the vascular bundles is the tissue in which berberine first makes its appearance and with increasing age of the organ penetration proceeds in a radial direction externally into the cortex and internally into the structural framework of the xylem elements. It was consistently found that old tissues possessed a high concentration of alkaloid, young actively growing shoots containing very low percentages.

Analysis of roots, main and subsidiary stems of plants 3, 5, 7 and 30 years of age throughout the year showed that the berberine content does not vary to an appreciable extent, the values in all plants examined remaining comparatively constant from October until the following March, when a definite rise took place and continued throughout the summer months until August when analyses were discontinued. As the plant increases in age it is apparent that accumulation of alkaloid takes place in the specified tissues from year to year. Moreover, during the winter and spring months no utilisation appears to take place, a fact which indicates that berberine cannot be considered as a reserve food material, which is deposited in the roots and rhizomes for use when growth processes are resumed. The gradual increase recorded during the summer months coincides with the period of most active growth, when the assimilatory area of the plant is increasing rapidly. In the interpretation of the foregoing results cognisance is taken of the effect of variations in proportions of alkaloid-containing tissue to the total amount of tissue included in the sample. For example in portions of the stem where much secondary thickening has taken place and where the proportion of wood to the richest berberine-containing tissue is large there would be a tendency for low values of alkaloid to be recorded, and the reverse would hold good for tissues such as parenchymatous etiolated shoots, where the portion of berberine (by weight) to the dry weight of the whole organ would be comparatively large. In these experiments, although the older plants possessed much greater bulk, the values for alkaloid percentages showed a steady rise with age, and errors which may have arisen from variations in the proportion of berberine-containing tissues to the total tissue of the organ do not vitiate the conclusions of the experiments. The facts of distribution give considerable support to the view that in *Berberis Darwinii*, the alkaloid is a product of metabolic activity which once formed does not again enter into the nutritive cycle of the plant.

PART II.

Cultural experiments.

Seven, 5 and 3-year-old plants, after samples of the stems and subsidiary roots had been taken, were potted in coarse river sand which had been thoroughly washed free from fine sediment, and watered at fortnightly intervals with one of the following culture solutions. Each solution (8 litres), contained 2 g. of magnesium sulphate, a trace of ferric chloride and (except (4)) 2 g. of potassium dihydrogen phosphate; with the addition of (1) 6 g. of calcium nitrate and 2 g. of potassium nitrate; (2) 6 g. of ammonium nitrate and 2 g. of calcium nitrate; (3) 2 g. of potassium nitrate and 4 g. of ammonium nitrate; (4) 6 g. of ammonium nitrate and 2 g. of calcium phosphate; (5) 2 g. of calcium phosphate; (6) 2 g. of calcium phosphate and 8 g. of asparagine; (7) 2 g. of calcium phosphate and 8 g. of urea; (8) as (5), with 1 litre of 1 % solution of formamol; (9) as (1) with 20 g. of glucose. The results of the culture experiments, which are described in detail below, are given in Tables V and VI.

The effect of different sources of nitrogen on the yield of berberine.

Potassium and calcium nitrates together as source of N (*Solution 1*). The results of the application of this solution to plants of 4, 5 and 7 years of age for the period of 9 months from November 1931 to August 1932 are set forth in Table V.

Table V. *Culture experiments with various sources of nitrogen.*

Berberine contents g./100 g.

			Control samples			After experiment		
Culture solution	Plant	Age	Root	Main stem	Sub-sidiary branches	Root	Main stem	Sub-sidiary branches
(1) Containing inorganic nitrate	(*a*)	4	1·58	0·27	—	1·57	0·21	—
	(*b*)	5	1·56	0·37	—	1·48	0·74	—
	(*c*)	7	2·5	0·69	0·13	2·01	0·69	0·13
(9) Containing inorganic nitrate with glucose	(*a*)	3	1·31	0·40	0·13	1·56	0·77	0·22
	(*b*)	3	1·19	0·23	0·11	1·21	0·32	0·19
(2) Excess of ammonium nitrate	(*a*)	7	1·57	0·64	0·13	1·66	0·45	0·13
	(*b*)	7	1·94	0·43	0·11	1·83	0·34	—
Do. with glucose	(*c*)	7	1·86	0·40	0·09	2·05	0·78	—
	(*d*)	5	1·52	—	—	1·60	0·39	—
(6) Asparagine as source of N	(*a*)	7	1·22	0·36	0·08	1·62	0·58	0·16
	(*b*)	5	1·63	0·27	—	1·85	0·35	—
(7) Urea as source of N	(*a*)	7	1·69	—	0·11	1·68	0·47	0·10
	(*b*)	5	1·45	0·16	0·03	1·66	0·24	0·10
(8) Formamol as source of N	(*a*)	3	1·22	0·41	—	1·84	0·35	—
	(*b*)	3	1·25	0·37	—	1·56	0·54	—

Solution 1 containing a preponderance of the nitrates of calcium and potassium was given to three 7-year-old plants. From November until March no new growth was made, but from March to August the plants produced strong healthy shoots, masses of fibrous roots and flowered profusely.

With the exception of the main stem of (*b*), organs of the plants to which excess of nitrates was applied show a slight decrease in the amount of alkaloid present. This result is the more significant when compared with the results in Table II above, which showed that plants growing in ordinary garden soil accumulated berberine during the summer months. This point will be discussed later.

Glucose in addition (Solution 9) was used with two 3-year-old plants from February to August 1931. Growth was strong and the plants flowered twice during the season. This addition has caused a definite increase in the berberine content of both roots and stems of plants 3 years of age. The increase is slightly greater than could be accounted for by accumulation during the summer months in normal untreated plants, a fact which points to the conclusion that the addition of glucose to nitrate solutions causes the production of larger absolute amounts of berberine than is usual in untreated plants.

Ammonium nitrate as source of N (*Solution 2*). Three plants of 7 years (*a*, *b*, *c*), and one of 5 years (*d*) in age were watered with this solution for a period of 8 months extending from the beginning of January until the end of August. Previously plants (*c*) and (*d*) had received glucose from August to October. Strong rank growth commenced in April and continued until the plants were examined in August. Two of these plants (*c*) and (*d*) which had received glucose did not produce flowers, one (*a*) produced flower buds which aborted, while the remaining plant (*b*) flowered late but profusely. Fibrous root development was excessive. The ammonium nitrate is not responsible for increased berberine production in roots as the slight rise indicated in the table is normal for plants of this age.

In two plants (*a*) and (*b*) the concentration in the main stems showed a definite decrease, while the stems and roots of (*c*) and (*d*) showed an increase, but it is significant that the latter plants were grown in a shaded situation and had received glucose.

The interpretation of these cultural experiments is difficult. The results with the application of the nitrates alone might be explained on the basis that the profuse growth resulting caused the utilisation of all the carbohydrate and nitrate available for protein synthesis, the carbohydrate being the limiting factor. When glucose is also applied however, there is an excess of both nitrate and glucose available, with possibly a production of amino-acids in excess of those required for protein synthesis and a resulting removal of this excess by increased berberine production. Such reasoning as this would entail that berberine synthesis would occur in those conditions in which soluble nitrogen and carbohydrate were available in excess of the immediate requirements of the plant. In this connection, it is to be emphasised that during the active growing period under the normal conditions there is a regular increase in berberine content from year to year.

Asparagine as the sole source of nitrogen (*Solution 6*). Two plants, (*a*) 7 years and (*b*) 5 years of age were given the asparagine solution during the periods January to August 1932 and April to August respectively. Both plants made vigorous growth and flowered freely. Of the two plants taken for experiment, the 7-year-old plant was noteworthy on account of a low alkaloid percentage in both root and stem. The effect of asparagine on this plant was to cause a very definite increase in berberine content in all parts, far greater than could be accounted for by annual accumulation. The 5-year plant did not show as great an increase, which might partly be accounted for by its much smaller leaf area. (One 7-year-old plant died before experimental samples were taken, due to absence of fibrous root development).

The berberine increase observed is due directly to the asparagine and it might occur in one of two ways. Asparagine yields aspartic acid on deamination and it is not unreasonable to suppose that on deamination this might yield malic acid. The production of this malic acid from an external source would possibly entail an economy of amino-acids or carbohydrate from which it would normally be formed, and cause an increased berberine production. On the other hand, asparagine or aspartic acid may be utilised directly in the building up of the alkaloid.

Urea as sole source of nitrogen (*Solution 7*). Samples of the root and stem of 7-year and 5-year-old plants were taken in April 1932 and the plants were watered with urea solution until the end of August. During this period both plants showed free healthy growth and flowered. Although growth was prolific the alkaloid content was not increased. The slight increase which occurred in plant (*b*) was not sufficient to show that urea acts differently from ammonium nitrate in its influence on alkaloid production, for this slight rise would have taken place in normal circumstances.

Formamol as sole source of nitrogen (*Solution 8*). Two 3-year-old plants were treated from August 1931 to July 1932. Growth was very slow and flowers were not produced. The application of formamol led to a definite increase in the berberine content of the roots of both plants under experiment. The stem of (*b*) also showed an increase, while that of (*a*) showed a fall in concentration of alkaloid which may be accounted for by assuming that during the period of experiment downward translocation was taking place into the rootstock.

The application of formamol in an acid soil is equivalent to the presentation of formaldehyde and ammonium citrate. The plants to which this substance was applied showed very little growth and produced no flowers, yet analysis of their roots and stems gave high results for the concentration of berberine. Here then a significant increase occurred with very restricted growth. These experiments however have in common with the ones already described, in which an increase in berberine resulted, the fact that in every case there was provided a carbohydrate or possible carbohydrate intermediate (glucose, succinic acid, citric acid) along with nitrogen (nitrate, asparagine, ammonia). It might be argued that in the formamol experiments, because growth was very restricted, there would be little protein synthesis and that in these plants protein breakdown would preponderate because the leaves were ageing. This being so, an increase in berberine might be expected to arise from the breakdown products and the effect of the formamol treatment was merely to increase the berberine by limiting growth in this manner. On the other hand, the results from experiments in which little or no growth occurred because of deprivation of calcium or potassium must be borne in mind, because in these experiments no such increase occurred.

This group of experiments may be summarised therefore by saying that increased berberine production has been observed only when there has been supplied to the plant a substance or substances which provide it with an external source of nitrogen and simple organic acids, and that the increase resulting is independent of growth.

Effects of withdrawal of essential elements.

Culture solution containing no nitrogen (*Solution 5*). Five 7-year (*a* to *e*) and two 3-year-old (*f* and *g*) plants were deprived of nitrogen for the following periods: (*a*) and (*b*) November 1931 to April 1932: (*c*) and (*d*) November 1931 to June 1932: (*e*) November 1931 to August 1932: (*f*) and (*g*) May to August 1932.

Table VI. *Culture experiments with nitrogen-free, calcium-free and potassium-free solutions.*

Berberine contents g./100 g.

				Control samples			After experiment		
Culture	Solution	Plant	Age years	Root	Main stem	Sub-sidiary branches	Root	Main stem	Sub-sidiary branches
(5)	No nitrogen	(*a*)	7	1·83	0·47	0·11	2·22	0·86	0·17
		(*b*)	7	2·45	0·36	0·07	2·15	0·40	0·11
		(*c*)	7	1·96	0·46	0·09	2·34	0·29	0·10
		(*d*)	7	1·65	0·41	0·11	1·66	0·42	0·11
		(*e*)	7	1·81	0·39	0·13	2·02	0·39	0·12
		Mean values		**1·94**	**0·42**	**0·10**	**2·07**	**0·47**	**0·12**
		(*f*)	3	1·13	0·24	—	1·26	0·24	—
		(*g*)	5	0·99	0·16	—	0·97	0·18	—
		Mean values		**1·06**	**0·20**	—	**1·11**	**0·21**	—
(3)	No calcium	(*a*)	3	1·43	0·19	—	1·59	0·21	—
		(*b*)	5	1·81	0·22	—	1·96	0·32	0·11
(4)	No potassium	(*a*)	3	0·84	0·20	—	0·86	0·21	—
		(*b*)	3	0·87	0·17	—	0·88	0·17	—

In all cases no growth took place, and during the winter the leaves of plants (*a*), (*b*) and (*c*) turned yellow and were cast. No young leaves appeared in spring and the plants became moribund and diseased. Plants (*d*), (*e*), (*f*) and (*g*) retained their leaves but made no growth.

Although the results of these experiments in the absence of nitrogen are somewhat erratic, ample proof is given that the alkaloid value does not fall even when conditions are optimum for utilisation of all available nitrogen. The figures in some cases show an increase during the time the plant is drawing upon its nitrogenous reserves, indicating that in conditions which appear to be unfavourable to the synthesis of alkaloid, its production still persists. These observations permit the conclusion to be drawn that the plant is unable to utilise the nitrogen of its alkaloid even when conditions are most favourable. The experimental results of Mothes [1928] showed that in *Nicotiana*, nicotine was still synthesised in conditions unfavourable to the growth of the plant. During nitrogen starvation, the nitrogenous reserves in roots, rhizomes and stems would be drawn upon for re-synthesis of protein at the growing points, until eventually complete exhaustion would take place, after which all soluble nitrogen would be derived from the breakdown of older leaf proteins. It seems remarkable that even in these circumstances the synthesis of berberine should still continue, and again it is apparent that there exists a parallelism between the synthesis of the alkaloid and the degree of protein breakdown prevailing.

Culture solution containing no calcium (*Solution 3*). From April to August 1932 one 3-year-old (*a*) and one 5-year-old plant (*b*) were deprived of calcium with the result that during the season no leaf or shoot growth took place, although flowers were produced in abundance. Deprivation of calcium, while checking the production of new growth did not seem to affect the seasonal rise in berberine content. The slight rise in concentration recorded in both root and stem has been shown to take place in plants growing in normal conditions.

Now calcium is considered to be an essential factor in the mechanism of protein synthesis and also in the building up of the cell wall, so that plants deprived of it usually show stunted growth and predisposition to disease. In the present experiment although the plants did not produce new growth they remained quite healthy, and analyses showed that during the period of experiment the concentration of berberine increased by an amount compatible with the normal rise during the summer months. During this period protein degradation would proceed normally, but the intermediates would not be capable of recombination to form protein at the growing points as lack of calcium inhibits this re-synthesis. The soluble nitrogen it is presumed would show a high value and a rise in alkaloid was observed.

Culture solution containing no potassium (*Solution 4*). During the same period as in the calcium experiments, two 3-year-old plants were watered with potassium-free solution. Again no new growth was observed, and one only (*a*) of the two plants flowered. The alkaloidal content of plants grown in the potassium-free medium remained unaffected as compared with controls, but growth processes being entirely checked, the rise in berberine content during the summer months was not so pronounced as under normal conditions.

Since potassium is considered to be an important factor in carbohydrate synthesis and translocation there would be a tendency towards carbohydrate deficiency in potassium-starved plants. From the experiments the evidence that carbohydrate concentration is of great importance in the determination of berberine concentration is convincing, and the experimental conditions of these plants would be similar to those in the set of experiments on the effect of nitrates without addition of carbohydrate. It appears that alkaloid concentration does not rise because there is a deficiency of carbohydrate in relation to the amount of available nitrogen.

Part III.

Effects of etiolation.

One plant (*a*) 7 years of age and 5 plants (*b* to *f*) 3 years of age were potted in sand, placed in complete darkness for varying periods and watered at fortnightly intervals with culture solutions 1 and 2, (*a*) and (*c*) receiving solution 2 (ammonium nitrate) and (*b*), (*c*), (*e*) and (*f*) solution 1 ($Ca(NO_3)_2$ and KNO_3). The results are recorded in Table VII.

Table VII. *Effects of etiolation.*

Berberine content g./100 g.

		Before experiment			After experiment			
Plant	Period of experiment	Root	Main stem	Subsidiary branches	Root	Main stem	Subsidiary branches	Etiolated shoots
(*a*)	Mar.–Aug.	2·06	0·43	0·14	2·07	0·53	0·26	1·5
(*b*)	May–Aug.	0·61	0·20	—	1·42	0·31	—	1·36
(*c*)	April–Aug.	1·13	0·23	—	2·12	0·50	—	1·2
(*d*)	June–Aug.	0·83			1·25	—	—	0·85
(*e*)	June–Aug.	0·97	0·13		—	—	0·05 *	0·72
(*f*)	May–Aug.	1·12	0·17	—	1·98	0·29	—	1·2

* Young shoots.

(*a*), (*b*), (*c*) and (*f*) cast their leaves and produced strong brilliant yellow shoots from the leaf axils and rootstocks, some of which attained a length of 18 inches, the average length being about 12 inches. Investigation of the anatomy of these etiolated shoots showed full development of vascular bundles with the surrounding ring of cells very rich in berberine. Plant (*d*) was ringed at a level of 6 inches from the surface of the sand. Etiolated shoots were not produced from the stem above the ring, but the plant put forth from the stem below the ring two strong shoots which contained a high percentage of berberine.

Plant (*e*) had produced young and strong shoots before being placed in darkness and these shoots showed rapid growth in the absence of light. It was observed that while the berberine concentration of that portion of the shoots which had grown under normal conditions was very low, the etiolated upper portions showed a high concentration.

Plant (*f*), after 3 months' growth in the dark was exposed to full light. Chlorophyll gradually appeared in the etiolated shoots which lost their yellow colour. Etiolated shoots of (*f*) after exposure to light for 2 weeks showed a berberine percentage of 0·47. Histological examination showed a disappearance of berberine from the upper portions of the shoots while the lower portions remained rich.

From Table VII it is apparent that growth in darkness is responsible for a marked increase in the concentration of berberine in all parts of the plant which were examined. The etiolated shoot was found to show a very high concentration in relation to the age of the tissue, and comparison with the values of young normal shoots reveals the fact that the influence of light is a very important factor in the production or location of the alkaloid. This result is in agreement with that of Ripert [1921] for the atropine and hyoscyamine contents of *Atropa*. The question thus arises as to whether the berberine which appears in such concentration in the etiolated shoot of *Berberis* is the result of translocation from the stem and root or whether it is synthesised *in situ* during the rapid growth of the shoot. Consideration of the behaviour of plants (*d*), (*e*) and (*f*) affords support to the supposition that the alkaloid is produced in the actively growing etiolated tissue. Those parts of the shoots of plant (*e*) which had grown in normal conditions of light intensity showed very low concentrations of alkaloid, but in the region of the continued growth of the shoots after removal of the plant to darkness, the berberine concentration was found to be high, a fact which seems definitely to favour the view that alkaloid is produced in the growing tissues of the etiolated shoot. Plant (*d*) was ringed 3 inches above the soil surface and placed in darkness. No growth took place above the ring, but shoots which contained a high percentage of berberine were produced from the stem below the ring. As the removal of bark would prevent any downward translocation of berberine, the high value attributed to the shoots can only be explained as due to either synthesis in the region of growth or translocation upwards from the stem and root. Examination of the behaviour of etiolated shoots of plant (*f*) when exposed to light tended to confirm the view that the high concentration of alkaloid in the etiolated shoot is due to synthesis in the growing region and not to translocation, as the extended apical growth region (where growth had taken place in presence of light), showed practically an absence of berberine.

Rhizomes which had appeared above the ground (as shoots) presented an interesting appearance on histological examination, which revealed the existence of an abrupt fall in berberine content in the region of the stem immediately above the ground. In the portions of rhizome below the surface of the soil, cells of the cortex and xylem appeared rich in alkaloid but at the level of half an inch above the surface, the corresponding tissues of the young stem were practically devoid of berberine. This observation strengthens the assumption that the alkaloid chiefly accumulates in tissues which are not exposed to light.

These experiments on the effects of etiolation appear to justify the deduction that the connection between protein breakdown and alkaloid synthesis is very close, as increase of the former appears to lead to increase in the amount of alkaloid produced. With regard to the region of synthesis of berberine it is suggested that this takes place in the etiolated shoot itself from products of protein breakdown and sugar residues translocated upwards from the stems and roots (*cf.* plant (*d*)).

PART IV.

Ringing experiments.

In the following experiments a narrow ring of bark was removed from the main stem at a level of 1 ft. from the ground. The ring was vaselined and bound with tape. At the end of the periods stated analyses of the root and portions of the stem above and below the ring were made. The results are set out in Table VIII.

Table VIII. *Ringing experiments.*

Berberine content g./100 g.

Plant	Period of experiment	Root conc.	Stem below ring	Stem above ring
(*a*)	May 1931–Oct. 1931	0·37	0·22	0·08
(*b*)	May 1931–Oct. 1931	0·31	0·23	0·13
(*c*)	Aug. 1931–Jan. 1932	1·21	0·36	0·21
(*d*)	Oct. 1931–Mar. 1932	0·88	0·21	0·18
(*e*)	Jan. 1932–June 1932	1·20	0·27	0·15
			Mean 0·26	0·15
(*f*)	Mar. 1932–Aug. 1932	1·83	0·94	0·48

Leaf and shoot growth on the stem below the ring was normal; above the ring no growth took place, the leaves assumed a pale green colour in the spring and summer but in September rapidly turned brilliant red.

In all plants except one (*d*) young shoots and leaves were removed from the stem below the ring as quickly as they appeared. The shoots and leaves which appeared below the ring in the case of (*d*) were allowed to remain.

Analysis of leaves above the ring revealed the presence of large amounts of tannin and carbohydrates, the latter including both reducing and non-reducing sugars, while the stem below the ring was relatively poor in these substances. In two instances (*a*) and (*b*) root development was completely inhibited and the root bark cast, and in the remaining plants fibrous root development was lacking.

The mean values for stems of the 3-year-old plants (*a*, *b*, *c*, *d*, *e*) show a high figure for alkaloid values above the ring when compared with figures for the corresponding tissues of unringed plants of this age (compare Table I, subsidiary branches, 3-year-old plants).

This fact is borne out by histological examination. Possible translocation of alkaloid in a downward direction therefore appears to be checked by the removal of the ring of bark.

The recent work of Maskell and Mason [1928; 1929] on the paths of translocation of food materials in plants has established the fact that the phloem is the tissue through which elaborated carbohydrate and nitrogenous substances pass in both upward and downward directions. Removal of a ring of bark from the stem would therefore impede the translocation of these substances to and from the root and rhizomes. The plants on which this experiment was made quickly lost their capacity for growth, and the leaves on the branches above the ring showed ageing. It was observed that in all tissues above the ring, accumulation of carbohydrate, tannins and anthocyanins took place, and, although analysis of these tissues for nitrogenous substances was not carried out, it is reasonable to assume that they would also accumulate in this circumstance. After the experimental period the roots in most cases gave evidence of starvation, casting of bark took place, and fibrous root development was completely inhibited. Analysis of parts above and below the ring gave varying results. The roots of plants in which decortication had taken place naturally were poor in alkaloid, while the mean values for the portions of stems above the ring were higher than the mean of this tissue in normal plants. Root and stem concentrations otherwise were normal for plants of this age. Unfortunately these experiments do not definitely elucidate the problem of the possible translocation of berberine, as the high mean value of the stems above the ring may be explained as being due either to obstruction of the alkaloid in downward movement or to an increased production, the result of the accumulation of carbohydrate and protein degradation products in the upper parts. Absence of fibrous root development would cause a deficiency of inorganic nitrogen in the upper region of the plant, as absorption of water could not take place to any extent. It would therefore be expected that the demand for soluble nitrogen in the tissues above the ring would be partially satisfied by disruption of the elaborated protein of leaves and stems. Carbohydrates being abundant, conditions favourable for the synthesis of berberine would be realised. Satisfactory conclusions regarding the transportation of berberine cannot be drawn from the results of these experiments and further work is necessary before the problem of the exact mode and place of berberine synthesis can be resolved.

SUMMARY.

1. In *Berberis Darwinii* the alkaloid berberine appears to be a waste product of metabolism as suggested by Pictet, for it accumulates in tissues which take no part in metabolic activities, and which are made up principally of dead elements. Synthesis of the alkaloid takes place in summer during the period of active growth, and no decomposition occurs during the winter or spring, so that the absolute amount present in the plant increases year by year.

2. The application of asparagine as a nitrogenous fertiliser leads to an increase in berberine content, whereas with ammonium salts and nitrates glucose must be given in addition. Growth on a nitrogen-free medium to the point of exhaustion does not bring about the decomposition of berberine, the normal increase with age was, in fact, observed, suggesting that synthesis of the alkaloid is connected with protein decomposition.

3. Etiolation leads to the production of shoots rich in berberine. If the bark be ringed these shoots appear only below the ring, suggesting that the berberine is synthesised from products translocated upwards from the root system.

4. Ringing experiments with plants grown in the light show an accumulation of berberine above the ring, suggesting that synthesis has taken place from products that are normally translocated from the leaves downwards to the root system.

5. Although the evidence is not conclusive, it appears that berberine is definitely a product of protein metabolism. It is suggested that synthesis is brought about by condensation of the products of protein breakdown and sugar residues.

The author wishes to record his indebtedness to Prof. Channon for his continued help and encouragement during the course of this work, and to acknowledge the kindness of Prof. J. McLean Thompson of the Department of Botany for supplying the *Berberis* plants used.

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CXIII. THE NEED FOR A STANDARD OF REFERENCE IN VITAMIN A TESTING.

BY KATHARINE HOPE COWARD, KATHLEEN MARY KEY AND BARBARA GWYNNETH EMILY MORGAN.

From the Pharmacological Laboratories of the Pharmaceutical Society of Great Britain.

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INCREASING numbers of workers are realising that greater accuracy is obtained in growth tests for vitamins by considering, not the growth curves of single animals, but the mean growth curves of groups of animals [Chick and Jackson, 1932; Coward *et al.*, 1930; 1931; Sherman and Batchelder, 1931]. In this laboratory, the vitamin A content of any sample of cod-liver oil has been determined by finding the mean increase in weight in 5 weeks of a group of rats each of which was given the same daily dose of the oil after it had ceased to grow on a diet deficient in vitamin A. During the last two years, a simultaneous test of a particular sample of cod-liver oil serving as a standard of reference has been made with each sample of oil tested. Results obtained from the successive tests of this oil have indicated that the average response of groups of rats to the same amount of a given sample of cod-liver oil may fluctuate greatly from month to month, and some evidence of this has already been published [Coward, Dyer and Morton, 1932]. The examination of a series of cod-liver oils showed that the intensity of absorption at 328 $m\mu$ was the physical measurement which agreed best with the biological estimate of vitamin A, but for certain oils even this agreement was bad. The agreement was better when the relative vitamin A potency of different oils was estimated by simultaneous biological examination of the standard oil, rather than by examinations made at different times. Thus it was evident that in order to make biological estimates in terms of a common standard, such as the carotene recently proposed as an international standard, simultaneous tests of the unknown substance and the standard must always be made.

Further tests on the oil used as a standard of reference emphasise still more the need of a standard of reference in every estimation. In all, 13 tests have been made on the same oil, using the same technique, the same daily dose of the oil (2 mg.) and rats as nearly alike as our colony could supply. There has been no change in the laboratory workers. The variations in the apparent vitamin A value of this sample of cod-liver oil are given in Table I. To obtain each of the figures, about 10 rats (5 bucks and 5 does) were given a vitamin A-free diet until they became steady in weight. Each rat was then given a daily dose of 2 mg. of our standard oil for 5 weeks. From the curves of response drawn up in the laboratory, which relate mean increase in weight to dose of vitamin A given, the abscissae corresponding to the mean increases in weight of the groups of rats were determined. The average of the abscissae for the bucks and does in successive tests gives the relative potency of the oil at different times.

Table I. *Variations in the apparent vitamin A value of one sample of cod-liver oil in successive tests on different groups of about 10 rats.*

Date of test	Average of abscissae (weighted means) of curves of response (for bucks and does) relating mean increase in weight in 5 weeks of a group of rats to dose of vitamin A
June–July, 1931	0·93
August, 1931	1·05
October, 1931	1·18
November, 1931	0·73
Jan.–Feb., 1932	1·52
March, 1932	1·29
April–May, 1932	1·11
June, 1932	2·64
July–Aug., 1932	3·48
August, 1932	2·30
Aug.–Sept., 1932	1·33
September, 1932	2·03
Sept.–Oct., 1932	1·58

The first seven values were reported in the paper by Coward, Dyer and Morton [1932]. It was shown that the different values could be attributed to errors of random sampling, *i.e.* considering the standard deviation of the response of the animals, these variations could occur without change in the general conditions of the experiments. But in June, 1932, some change took place which induced in the rats a greater response to 2 mg. of the oil than had ever before been given. In the test made in July–August, 1932, the apparent value of the oil was five times its apparent value in November, 1931. It is unbelievable that the actual vitamin A value of the oil could increase. It must, therefore, be admitted that equal doses of vitamin A can bring about very different responses in groups of rats at different times. It follows that equal responses in groups of rats at different times do not prove that the doses given are of equal vitamin A potency. The need for making a simultaneous test on a standard of reference whenever a substance is tested for vitamin A is obvious.

(Experiments are in progress in many laboratories concerning the use of a particular sample of carotene as a standard of reference for vitamin A. This sample is issued by the National Institute for Medical Research, Hampstead, on behalf of the Permanent Standards Committee of the League of Nations.)

Possible causes of the variation in response to the same dose of vitamin A.

The variation in response to 2 mg. oil A was gradual, though it was evidently not seasonal (Table I). The differences in response may have been due to (*a*) differences in the vitamin A-free diet of the animals, (*b*) differences in the animals themselves, (*c*) the influence of some undetermined factor, possibly in the diet of the breeding colony.

(*a*) *Variation in vitamin A-free diet.* The only change in the constituents of the vitamin A-free diet that we can detect during the time that the 13 tests of oil A were being made, was in the caseinogen, but this does not account for the sudden rise in the apparent value of oil A in June, 1932. Special batches of "light white casein" are allocated to our use by the British Drug Houses and delivered as required, 1 cwt. at a time. One batch was exhausted and another set aside for us in June, 1932, but the June test on oil A was finished before the first hundredweight of the second batch was delivered to us in July. Thus, so far as we can tell, there were no variations in the caseinogen to account for the variations in the apparent vitamin A value of the oil. The amount of vitamin D given to the rats each week (8 units per rat) has not been changed, and from tests of the vitamin B content of several samples of dried yeast, though not of all, we think that the dried yeast has not varied appreciably during the tests on oil A. Thus it seems impossible to account for the variations in response to the same dose of oil A on the ground of changes in the basal diet of the rats.

(*b*) *Variations in the test animals.*

1. *The degree of correlation between the rate of growth in the preparatory period* (*i.e.* the time during which the rat is fed on a vitamin A-free diet only, until it ceases to grow) *and the growth during the 5 weeks* when the rat was given a daily dose of cod-liver oil has been determined. It had occasionally occurred to us that when a rat grows slowly in the preparatory period, its response to a given dose of vitamin A is slower than that of a rat which grows rapidly in the preparatory period. A measure of the rate of growth in the preparatory period was made by determining the increase in weight of the rat in the first 5 weeks of feeding on the vitamin A-free diet only. Most of the rats grew for 6 or 7 weeks before becoming steady in weight, so that the rate of growth during the first 5 weeks seemed to be a fair estimate of the "growth potential" of the rat. The degrees of correlation between the rate of growth in the preparatory period and the growth during the 5 weeks' test period for the bucks and does respectively were found to be:

$-$ 0·033 for the bucks (63);
$-$ 0·135 for the does (82).

These figures should be at least $+$ 0·208 and $+$ 0·183 respectively to indicate a positive correlation ($P = 0{\cdot}1$, *i.e.* the probability that a correlation should arise by random sampling from an uncorrelated population). The figure $-$ 0·135 for the does indicates approximately a 1 in 2 chance of a definite relationship, but as the sign is negative, it indicates that the greater the rate of growth in the preparatory period, the slower the rate of growth in the test period and *vice versa*. Thus, in this colony of rats, there is no indication that a greater rate of growth in the preparatory period of feeding on the vitamin A-free diet only is accompanied by a greater response to a dose of vitamin A when this is given to the rat.

2. *The degree of correlation between the weight of the rat at the beginning of the test period* (*i.e.* at the time the rat ceased to grow and was first given a dose of cod-liver oil) *and the increase in weight in 5 weeks* during which it was given a daily dose of 2 mg. of oil A has been determined. As we discard male rats which do not cease to grow before they attain a weight of 120 g. and female rats at 110 g. our investigation does not cover a very wide range of initial weights. It was found that:

$r = -\,0{\cdot}194$ as determined from 63 bucks;
$r = -\,0{\cdot}095$ as determined from 82 does.

For a probability of $P = 0{\cdot}1$ that there is no correlation, r would have to $= -\,0{\cdot}208$ ($n = 63 - 2$ bucks) to indicate that the higher the initial weight the less (on account of the *minus* sign) would be the response. Similarly, r for the does would have to be $= -\,0{\cdot}183$. Thus the probability that differences in weight at the beginning of the test period would affect the results under the conditions and limitations of these tests appears to be small.

When, however, the correlation between the initial weight of the rat and the abscissa of the curve of response corresponding to the increase in weight of the rat is determined, it is found that:

$$r = -\,0{\cdot}2472 \text{ for the bucks;}$$
$$r = -\,0{\cdot}2456 \text{ for the does.}$$

Whence $P = 0{\cdot}025$ for the bucks, and $P = 0{\cdot}05$ for the does.

This method of determining a correlation indicates that the initial weight of the rat influences the response. It may be a sounder method than the former, and it confirms the conclusion of Sherman and Burtis [1928] who made their calculation by a different method from either of those given in this paper.

Table II.

	♂			♀		
Test	Average weight at beginning of test period g.	Increase in weight in 5 weeks g.	Abscissa corre-sponding to increase in weight	Average weight at beginning of test period g.	Increase in weight in 5 weeks g.	Abscissa corre-sponding to increase in weight
1	95·1	11·4	1·00	75·0	9·25	0·77
2	91·3	9·3	0·90	76·3	14·5	1·20
3	90·0	16·1	1·24	91·0	13·7	1·12
4	76·7	7·8	0·85	79·7	6·7	0·62
5	98·6	20·6	1·53	81·0	17·4	1·52
6	—	—	—	78·4	15·4	1·29
7	69·7	14·7	1·17	67·7	13·3	1·08
8	87·6	29·8	2·33	83·5	25·5	3·01
9	73·0	42·0	4·08	91·2	26·0	3·14
10	84·0	19·6	1·46	88·4	26·0	3·14
11	94·7	8·0	0·86	88·4	18·8	1·71
12	73·7	26·3	1·99	57·9	20·9	2·04
13	77·9	18·1	1·36	70·1	19·1	1·76

We then compared the average initial weights of the groups of rats in the several tests with the average increases in weight in 5 weeks and also with the abscissae of the curve of response corresponding with these average increases in weight (Table II). It is, however, evident that differences in initial weight do not account wholly for the big differences in response of the different groups of animals to the same dose of vitamin A.

SUMMARY.

Thirteen tests of the vitamin A content of a particular sample of cod-liver oil have been made on groups of rats, each group containing 10–12 animals. The tests were made successively and extended over about 17 months. A five-fold variation in result was found.

The variation in result is not a seasonal one. It is apparently not due to changes in the basal diet. It is not due to variations in the weights of the rats when they were first given the doses of cod-liver oil, or to differences in rate of growth in the preparatory period during which the rats' reserves of vitamin A were being exhausted. The variation must, therefore, be due to some influence not yet determined.

It is obviously essential that the vitamin A content of a substance should be determined not simply by the response of a particular group of animals to a given dose of the substance, but by a comparison of the response of this group with the response of another group to a dose of some standard of reference, the two tests being carried out simultaneously.

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CXIV. VARIATIONS IN THE QUALITY OF BUTTER, PARTICULARLY IN RELATION TO THE VITAMIN A, CAROTENE AND XANTHOPHYLL CONTENT AS INFLUENCED BY FEEDING ARTIFICIALLY DRIED GRASS TO STALL-FED CATTLE.

By ALBERT EDWARD GILLAM, ISIDOR MORRIS HEILBRON
AND RICHARD ALAN MORTON,
Department of Chemistry, University of Liverpool,
AND
GERALD BISHOP AND JACK CECIL DRUMMOND,
Department of Physiology and Biochemistry, University College, London.

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THE natural diet of herbivora includes no vitamin A, but the carotene of green plants is transformed *in vivo* into the vitamin to an extent sufficient to provide for the needs of the animal. Milk from grazing cattle yields butter containing both vitamin A and carotene, the latter (with a small amount of xanthophyll) being responsible for the yellow colour. In the process of converting grass into hay for winter feeding, much of the carotene disappears, and in consequence winter butter is normally much paler than summer butter. A corresponding decrease in vitamin A content need not necessarily occur, since the lactating animal may utilise some of the vitamin A stored in the liver. It is important, therefore, that the vitamin A and carotene present in butter should be ascertained separately. Biological assay indicates the total growth-promoting effect, but this will be a summation of the contributions due to vitamin A and that fraction of the carotene which is transformed into the vitamin in the rat.

The spectroscopic method described by Morton and Heilbron [1930], whilst not differentiating between carotene and xanthophyll, provides a rapid means of measuring vitamin A content as well as total carotenoid content. By employing the phase separation between aqueous methyl alcohol and light petroleum [Kraus, 1872; Borodin, 1883; Willstätter and Stoll, 1913] the quantities of carotene, xanthophyll and vitamin A can now be determined separately, thus constituting a refinement of the earlier procedure. The method evaluates the carotene content irrespective of the efficiency with which the hydrocarbon can be utilised.

The scheme of investigation of which this work forms part involves a study of the variations in the quality of milk in relation to diet, which in turn involves the seasonal variations which occur in normal dairy practice. A general report on the organisation of the experiment and its broader implications will appear elsewhere [Watson, Drummond, Heilbron and Morton, 1933].

Four groups of cows were employed.

Group	Ration
1	Normal winter diet of hay, roots and purchased concentrates
2	Normal winter diet, hay replaced by medium quality grass silage
3	Normal winter diet, 75 % of concentrates replaced by artificially dried nitrogen-fertilised grass
4	As for 3 except that the grass had received no nitrogenous fertiliser dressing

Representative samples of milk were then taken at certain specified periods and butter made therefrom for the carotene-vitamin A analyses.

The treatment of the butters.

A 250 g. portion of each butter (the moisture content having been determined) was saponified for half an hour with a slight excess of 20 % alcoholic KOH. The product was poured into three times its volume of water and extracted with ether until the extract was colourless. The bulked extract was washed with a little water without shaking and then separated and washed with several portions of water, with shaking. The whole of the washed ether extract was reduced to small volume (about 10 cc.) in an atmosphere of nitrogen. The carotene and xanthophyll were then separated by the phase method as follows. 20 cc. of low-boiling light petroleum were added and the xanthophyll extracted from this solution by ten successive treatments with 85 % methyl alcohol, 300–350 cc. being used in all. The light petroleum solution, containing all the carotene, was washed with water, reduced to small bulk in an atmosphere of nitrogen and the concentrated solution transferred completely to an ampoule, where moisture and the remainder of the solvent were removed *in vacuo*, the residue being finally sealed off in nitrogen. The xanthophyll fraction in the combined methyl alcohol extracts was diluted with water and extracted with ether until free from yellow colour. The ether extracts were again mixed, washed and concentrated, transferred to an ampoule, evaporated and the residue sealed off in nitrogen as before. Thus two ampoules were available, the contents of which represented the whole of the carotene and xanthophyll respectively from 250 g. of fresh butter, the vitamin A being distributed between the two. By using ampoules of brown glass and sealing off in nitrogen, safe storage was ensured until the samples were required for analysis.

Before proceeding to report on the amounts of carotene, xanthophyll and vitamin A in the fractionated unsaponifiable matter of the butters it is necessary to refer to the standard spectroscopic data on the pure substances.

It has been established [Karrer, Morf and Schöpp, 1931; Heilbron *et al.*, 1932] that vitamin A is a colourless unsaturated alcohol characterised by a broad absorption band, free from fine structure, extending over the region 280–370 $m\mu$ approximately, with a maximum near 328 $m\mu$ in alcohol at an intensity $E^{1\%}_{1\,\mathrm{cm.}} = 1600$ [Carr and Jewell, 1933] (the molecular extinction coefficient calculated on $C_{20}H_{30}O/\overline{=}_5$ is probably close to 46,000 for alcoholic solutions). In any medium not exhibiting appreciable absorptive power at 328 $m\mu$, it is easy to determine quantitatively the proportion of vitamin A in solution by measuring the extinction at 328 $m\mu$ for a given solution and cell thickness. Vitamin A also gives rise to a blue solution with antimony trichloride in chloroform, characterised by a sharp absorption maximum at 617 $m\mu$ and a subsidiary band at 583 $m\mu$. For the apparently pure vitamin these bands have intensities which can be expressed as $E^{1\%}_{1\,\mathrm{cm.}}\ 617\,m\mu = 5000$ and $E^{1\%}_{1\,\mathrm{cm.}}\ 583\,m\mu = 2600$, the notation $E^{1\%}_{1\,\mathrm{cm.}}$ referring to $E = \log I_0/I$ (I_0 = intensity of incident light, I = intensity of emergent light) for a solution containing 1 % of material in almost saturated antimony trichloride in chloroform and viewed through a 1 cm. cell.

Carotene in chloroform exhibits absorption maxima at 495, 463, 436, 348 and $280 m\mu$. The intensity of absorption at the head of the main band in the visible $E^{1\%}_{1\,\text{cm.}}$ $463 m\mu = 1900$ ($e = 102{,}000$) [Dulière, Morton and Drummond, 1929]. With antimony trichloride, carotene gives rise to a blue solution with a clearly defined absorption band exhibiting its maximum at $585\text{–}590 m\mu$ with an intensity of absorption $E^{1\%}_{1\,\text{cm}}$ $585 m\mu = 420$ [Gillam *et al.*, 1932].

A specimen of xanthophyll (M.P. 193°), kindly supplied by Prof. Karrer, and probably consisting largely of "lutein" [Kuhn *et al.*, 1931], was found to give rise to the following absorption maxima: 485, 455, 425, 405, 340 and $272 m\mu$ in chloroform. The intensity of absorption of this sample at the head of the main band in the visible $E^{1\%}_{1\,\text{cm.}}$ $455\ m\mu$ is 1570 in chloroform solution. The nature of the solvent is important in this connection, as in alcohol-ether and in light petroleum the value is very much larger [McNicholas, 1931]. With the antimony trichloride reagent, xanthophyll gives a blue solution exhibiting absorption bands at 617 and $585 m\mu$, superimposed on general absorption. The respective intensities can be expressed as $E^{1\%}_{1\,\text{cm.}}$ $617 m\mu = 250$ and $585 m\mu = 192$ (for spectroscopic measurements on a number of carotenoids, *cf.* Euler *et al.* [1932]). The data on the pure substances are summarised in Table I.

Table I.

	Carotene	Xanthophyll	Vitamin A
Wave-length maxima (chloroform solutions)	495, 463, 436, 348, $280 m\mu$	485, 455, 425, 405, 340, $272 m\mu$	$328\text{–}330 m\mu$
Intensity of absorption $E^{1\%}_{1\,\text{cm.}}$ (chloroform)	At $463 m\mu = 1900$	At $455 m\mu = 1570$	At $328 m\mu = 1600$
Absorption bands with antimony trichloride	$585\text{–}590 m\mu$	617 and $585 m\mu$	617 and $583 m\mu$
Intensity of absorption in the blue solutions with antimony chloride $E^{1\%}_{1\,\text{cm.}}$	At $587 m\mu = 420$	At $617 m\mu = 250$ At $585 m\mu = 192$	At $617 m\mu = 5000$ At $583 m\mu = 2600$

The absorption spectra measurements in the visible and ultra-violet have been carried out photographically on two Hilger E 3 quartz spectrographs, one being used with a short-focus rotating-sector photometer with a tungsten underwater spark as light source, and the other with an iron-nickel arc in air and a long-focus sector photometer. The absorption spectra determinations on the blue solution with antimony trichloride were all made by means of a Hilger-Nutting visual spectrophotometer, using the technique already described [Heilbron, Gillam and Morton, 1931].

The analytical problem therefore resolved itself into (*a*) the determination of carotene in a material substantially free from other coloured substances, (*b*) the determination of xanthophyll in a material similarly free from extraneous colouring matter, and (*c*) the determination of vitamin A in the presence of carotene in one fraction, and of xanthophyll in the other fraction. Direct determinations of the intensities of the absorption bands characteristic of carotene and xanthophyll respectively in comparison with standard data on the pure substances afford a satisfactory means of evaluation. Vitamin A can be detected in the presence of carotenoids by decolorising the solution with the minimum quantity of adsorbent carbon, after which the $328 m\mu$ absorption band or the colour reaction with antimony trichloride serves to identify it. There is no certainty, however, that the vitamin will be wholly unadsorbed. If the carotene or xanthophyll content of a given material is already known (from the direct absorption spectrum in the visible) it is easy to calculate the intensity of absorption at 328$m\mu$ arising from a stated weight of the carotenoid by using the known absorption curves for the pure substances in the ultra-violet (Fig. 1).

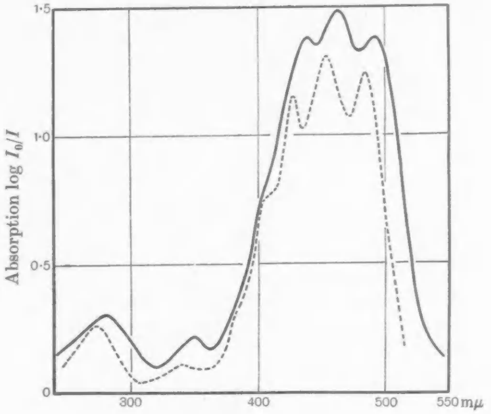

Fig. 1.

—— Carotene in chloroform, 0·00078 % 1 cm. cell.
- - - - Xanthophyll in chloroform, 0·0042 % 2 mm. cell.

By subtracting the calculated contribution of the carotenoid at 328$m\mu$ from the observed intensity at this wave-length, a reasonably accurate figure for the absorption due to vitamin A is obtained. The total vitamin A content of the butter is then obtained by adding together the observed differences in the carotene and xanthophyll fractions. In actual practice the contribution of the carotenoid to the gross absorption about 328$m\mu$ rarely exceeds 20 % in the carotene fractions, and in the xanthophyll fractions it is very much smaller.

The intensity of the blue colour developed on the addition of antimony trichloride to each of the fractions is measured at 617 and 585$m\mu$, the value at the latter wave-length as shown below being particularly useful. The spectroscopic blue value (at 585$m\mu$) due to carotene or xanthophyll can be calculated from the known carotenoid content as determined from the direct absorption spectrum, so that the difference between this calculated blue value and the observed one gives the blue intensity due to vitamin A. Two quantitative criteria for vitamin A, expressed in intensities of absorption, are thus obtained and it is necessary to calculate the amount of vitamin with which they correspond.

The ultra-violet method of assay is probably the most trustworthy for the determination of vitamin A, since the intensity of absorption is directly proportional to the amount of vitamin. On the basis of the spectroscopic data already given for pure vitamin A, the data have been calculated to percentages of vitamin. These are tabulated as the final values for the vitamin A content of the butters, but may need a slight constant correction throughout if and when vitamin A is isolated in a purer state.

The values obtained by the spectroscopic blue test provide approximate confirmation of the findings based on the ultra-violet method of measurement. When the antimony trichloride test is applied to liver oils (*i.e.* sources of vitamin A free from carotene and xanthophyll) the ratios of blue to ultra-violet values are not constant over a reasonable range or among vitamin A-bearing oils from various sources. This lack of constancy is due mainly to the presence of substances which inhibit the formation of the blue colour. Norris and Church [1930] have shown that the blue colour is not strictly proportional to the vitamin A potency in liver oils, and in fact that the addition of certain substances (such as oleic acid) inhibits its formation quite appreciably. If inhibiting substances are present in liver oils they appear to be removed, at least partially, by saponification, since Smith and Hazley [1930] have shown that after saponification there is a considerable increase in the recorded blue value. That the ratio of blue colour value to ultra-violet value is not constant even after removal of the saponifiable matter is shown by the increase in this ratio as the vitamin A is concentrated in the unsaponifiable matter by various treatments [Heilbron *et al.*, 1932; Heilbron, Gillam and Morton, 1931; Gillam and Morton, 1931]. Both the 617 and the 583 $m\mu$ bands are inhibited but the latter band is affected much less than the former [Emmerie *et al.*, 1931; Eekelen *et al.*, 1931; Morton, 1932]. For this reason and also because vitamin A, carotene and xanthophyll all show absorption maxima near 585 $m\mu$, the intensity measurements at this wave-length are to be considered more trustworthy than those at 617 $m\mu$ in the case of the butter samples.

Table II.

Sample dated	The vitamin A value in terms of the ultra-violet absorption expressed as $E^{1\,\%\ \text{fresh butter}}_{1\ \text{cm.}}$ at 328 $m\mu$				Spectroscopic blue value due to vitamin A expressed as $E^{1\,\%\ \text{fresh butter}}_{1\ \text{cm.}}$ at 585 $m\mu$			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
9. xii. 31	0·0086	0·0098	—	—	0·0105	0·0113	—	—
16. xii. 31	0·00745	0·00504	0·00697	0·0063	0·0072	0·00579	0·00874	0·009
12. i. 32	0·00545	0·00838	0·0110	0·0103	0·00791	0·0125	0·0162	0·0167
26. i. 32	0·00554	0·00725	0·0119	0·0093	0·0071	0·0091	0·0202	0·0146
9. ii. 32	0·00522	0·00415	0·0090	0·00637	0·00683	0·00577	0·0168	0·0117
26. ii. 32	0·00373	0·00243	0·00823	0·00658	0·00613	0·00323	0·0177	0·0121
16. iii. 32	0·00263	0·00314	0·00668	0·0067	0·0043	0·00395	0·0118	0·01033
6. iv. 32	0·00339	0·00303	0·00559	0·0040	0·00434	0·00421	0·0080	0·00534
19. iv. 32	0·00338	0·00224	0·00402	0·0043	0·00568	0·00322	0·00573	0·0086
10. v. 32	0·0093	—	0·0115	—	0·0153	—	0·0187	—
23. v. 32	0·0105	—	0·0113	—	0·0162	—	0·0205	—

Table II summarises the observed values of $E^{1\,\%\ \text{fresh butter}}_{1\ \text{cm.}}$ ($CHCl_3$) for vitamin A by both the ultra-violet absorption and the 585 $m\mu$ band in the colour test. The data show a general parallelism between blue and ultra-violet values. In the complete absence of inhibitors the ratio of ultra-violet values to 585 $m\mu$ blue values should be 1600:2600 (*i.e.* the relative intensities of the two bands in the purest vitamin A preparations).

Tables III, IV, V, and VI show all the values calculated for carotene, xanthophyll and vitamin A for each of the butters, expressed as mg. of substance per 100 g. of dry butter. The difference between the butters of the various groups of cows will be better appreciated from the graphs (Figs. 2, 3, 4, and 5).

The statement of Carr and Jewell [1933] that carotene cures xerophthalmia and supports growth in rats at the level of 0·001 mg. per rat per day, whereas their richest vitamin A concentrates ($E^{1\,\%}_{1\ \text{cm.}}$ 328 $m\mu = 1600$) are 1·66[1] times as

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[1] This is a conservative estimate of the superiority of vitamin A over carotene and has been used here pending agreement as to the relative growth-promoting effects of pure carotene and pure vitamin A.

Table III. *Group 1 butters.*

Cows receiving normal winter rations of roots, hay and concentrates.

Samples dated	Water %	Carotene in mg./100 g. on dry butter (1900 basis)	Xanthophyll in mg./100 g. on dry butter (1570 basis)	Vitamin A in mg./100 g. on dry butter (1600 basis)	Estimated growth-promoting activity in international units per g. (dry)
26. xi. 31	15·7	0·613	0·72	0·637	16·7
16. xii. 31	15·1	0·505	0·0180	0·549	14·2
13. i. 32	18·0	0·237	0·0124	0·416	9·3
26. i. 32	21·0	0·167	0·0105	0·438	9·0
9. ii. 32	15·0	0·137	0·0097	0·384	7·77
1. iii. 32	15·3	0·070	0·0127	0·276	5·3
16. iii. 32	24·7	0·0828	0·0106	0·218	4·46
6. iv. 32	18·14	0·0674	0·00824	0·259	5·0
19. iv. 32	17·5	0·0606	0·0069	0·256	4·88
10. v. 32	14·9	0·515	0·0360	0·683	16·5
23. v. 32	14·7	0·567	0·0469	0·770	18·5

Table IV. *Group 2 butters.*

Cows receiving normal winter rations of hay, roots and concentrates, except that the major part of the hay was replaced by grass silage.

Sample dated	Water %	Carotene in mg./100 g. on dry butter (1900 basis)	Xanthophyll in mg./100 g. on dry butter (1570 basis)	Vitamin A in mg./100 g. on dry butter (1600 basis)	Estimated growth-promoting activity in international units per g. (dry)
26. xi. 31	14·9	0·521	0·0747	0·720	17·2
16. xii. 31	14·0	0·344	0·0148	0·367	9·56
12. i. 32	17·6	0·327	0·0216	0·635	13·85
26. i. 32	13·75	0·244	0·0081	0·544	11·5
9. ii. 32	17·8	0·167	0·0085	0·315	6·92
1. iii. 32	14·0	0·0868	0·0103	0·176	3·80
16. iii. 32	17·1	0·0776	0·0107	0·238	4·75
6. iv. 32	19·2	0·0585	0·0077	0·234	4·48
19. iv. 32	16·8	0·0465	0·00685	0·168	3·26

Table V. *Group 3 butters.*

Cows receiving normal winter diet, except that the concentrates were largely replaced by artificially dried nitrogen-treated grass (30 % concentrates, 70 % dried grass).

Samples dated	Water %	Carotene in mg./100 g. on dry butter (1900 basis)	Xanthophyll in mg./100 g. on dry butter (1570 basis)	Vitamin A in mg./100 g. on dry butter (1600 basis)	Estimated growth-promoting activity in international units per g. (dry)
16. xii. 31	13·8	0·490	0·00963	0·506	13·3
12. i. 32	16·0	0·502	0·033	0·818	18·7
26. i. 32	16·6	0·538	0·0412	0·888	20·2
9. ii. 32	19·2	0·521	0·0371	0·696	16·8
1. iii. 32	12·2	0·464	0·0319	0·586	14·4
16. iii. 32	17·8	0·496	0·0319	0·508	13·4
6. iv. 32	13·5	0·289	0·0074	0·404	9·62
19. iv. 32	14·7	0·119	0·00375	0·294	6·09
10. v. 32	15·0	0·495	0·0360	0·846	19·0
23. v. 32	15·0	0·703	0·0629	0·829	20·8

effective, makes it possible to express the total growth-promoting activity of a butter due to carotene *plus* vitamin A in terms of the international carotene standard (1 unit = 0·001 mg. of carotene).

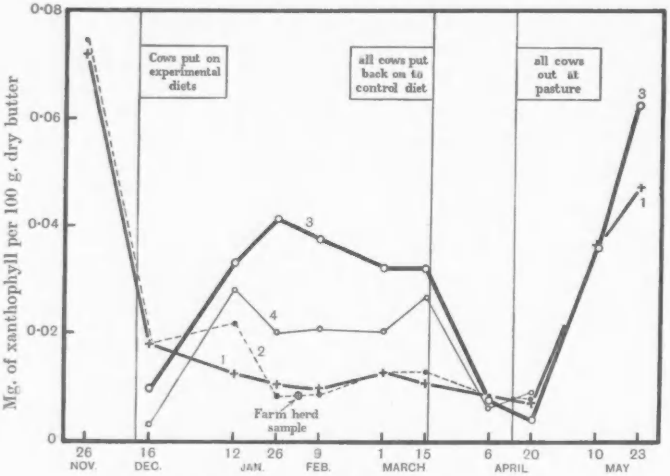

Fig. 2. Xanthophyll of the butters.

Group 1 +——+; 2 ●---●; 3 ○——○; 4 ○——○.

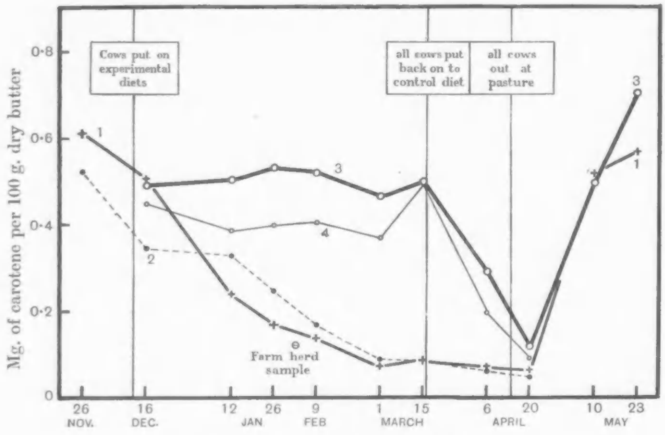

Fig. 3. Carotene of the butters.

Group 1 +——+; 2 ●---●; 3 ○——○; 4 ○——○.

The following example illustrates the method. A butter contains 0·703 mg./100 g. carotene and 0·828 mg./100 g. vitamin A on dry weight: 0·703 mg. carotene per 100 g. of dry butter fat equals 7·03 international units of carotene per g.;

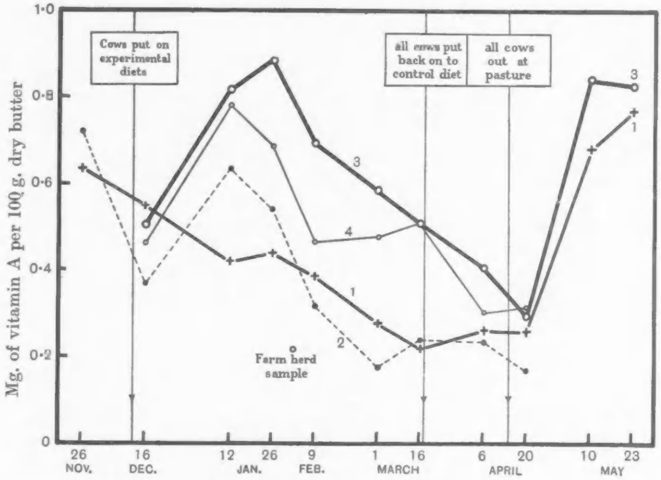

Fig. 4. Vitamin A of the butters.

Group 1 +——+; 2 ●- - -●; 3 ○——○; 4 ○——○.

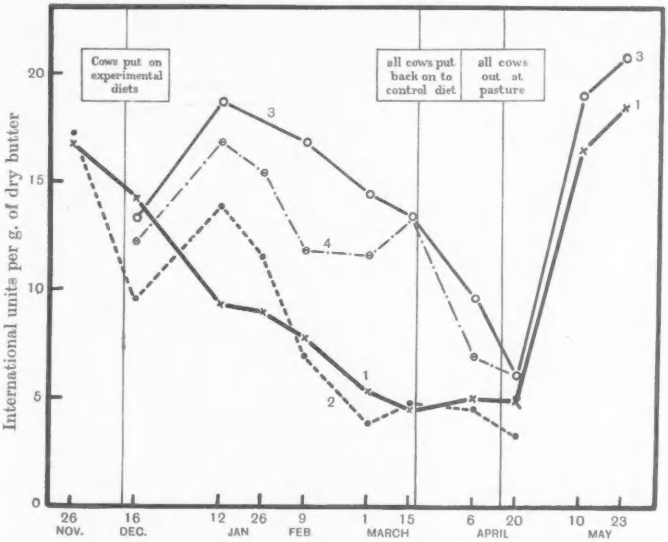

Fig. 5. Estimated total growth-promoting activity of the butters.

Group 1 ×——×; 2 ●- - -●; 3 ○——○; 4 ⊖—·—⊖.

Table VI. *Group 4 butters.*

Cows receiving normal winter rations, except that artificially dried non-nitrogen-treated grass replaced 70 % of the concentrates in the diet.

Samples dated	Water %	Carotene in mg./100 g. on dry butter (1900 basis)	Xanthophyll in mg./100 g. on dry butter (1570 basis)	Vitamin A in mg./100 g. on dry butter (1600 basis)	Estimated growth-promoting activity in international units per g. (dry)
16. xii. 31	14·6	0·447	0·003	0·462	12·2
12. i. 32	17·8	0·385	0·028	0·780	16·8
26. i. 32	15·3	0·398	0·022	0·685	15·4
9. ii. 32	14·1	0·407	0·0205	0·463	11·8
1. iii. 32	13·6	0·367	0·0301	0·475	11·6
16. iii. 32	17·8	0·491	0·0263	0·508	13·3
6. iv. 32	17·1	0·193	0·0062	0·301	6·95
19. iv. 32	14·1	0·0882	0·0088	0·312	6·08

Table VII.

Date of cutting grass			Carotene			Xanthophyll			
			Mg./100 g. by yellow colour (Lovibond)	Mg./100 g. by direct absorption spectrum	Mg./100 g. by spectroscopic colour test $SbCl_3$ 585 $m\mu$	Mg./100 g. by yellow colour (Lovibond)	Mg./100 g. by direct absorption spectrum	Mg./100 g. by $SbCl_3$ colour test (spectroscopic)	
								617 $m\mu$	585 $m\mu$
1. vii. 31	Rye grass field	A	22	15·0	18·0	35·2	18·1	48·3	53·4
,,	,,	B	24·3	—	—	38·2	40·0	79·7	85·5
15. vii. 31	,,	A	42·4	33·3	46·4	88·6	62·1	140	147
,,	,,	B	47·2	38·8	51·3	90·7	81·1	142	150
29. vii. 31	,,	A	37·2	25·3	43·7	71·8	56·8	119	127
,,	,,	B	51·9	38·9	46·8	102·2	89·4	177	193
16. ix. 31	,,	A	31·3	35·7	42·6	73·3	80·0	138	167
,,	,,	B	30·5	33·9	36·8	70·3	84·8	180	222
15. vii. 31	Clover grass field	A	33·01	25·1	38·4	48·5	43·2	71·3	69·0
,,	,,	B	33·08	28·5	36·0	48·9	52·8	90·0	94·7
29. vii. 31	,,	A	37·44	24·8	31·8	74·95	58·0	111·2	120
,,	,,	B	40·0	33·9	36·0	75·9	76·6	122	130
16. ix. 31	,,	A	51·5	46·1	53·8	106·1	87·3	210	212
,,	,,	B	51·7	42·4	56·3	107·1	101	189	218

0·829 mg. of vitamin A per 100 g. of fat equals 1·66 × 0·00829 mg. = 0·01381 mg. of carotene per g. Hence the total (estimated) growth-promoting activity of this particular butter = 13·81 + 7·03 = 20·84 international units per g.

In the Tables will be found the growth-promoting value of all the butters calculated in this way. The results are also shown graphically. In connection with the Group 2 butters it should be noted that the tower silage was exhausted on January 20th and was replaced by stack silage. This appears to have affected the vitamin A content adversely (*cf.* Fig. 4).

Note on the unsaponifiable matter of grass.

In a study of the carotenoid content of grass it has been found that the apparent percentages of carotene, as measured by the spectroscopic antimony trichloride colour test, although in fair agreement with the values based on direct absorption spectra measurements, reveal, on closer examination, a discrepancy in the sense that the colour test values are on the average 1·3 times those based on direct absorption spectra. A similar but greater discrepancy occurs in the xanthophyll determinations, the ratio in this case being approximately 2·2 (see Table VII).

The discrepancies are under detailed investigation, but it is already possible finally to rule out the plausible suggestion that they are due to the presence in grass of the vitamin A of liver oils. It seems much more likely that the carotenoids of grass include substances other than "carotene" and "xanthophyll," accepting the criteria of carotene and xanthophyll given earlier in this paper. The principal contaminant appears to accompany xanthophyll in the phase separation and may be the xanthophyll analogue of γ-carotene which contains only one ionone ring [Winterstein, 1933; Kuhn and Brockmann, 1933].

The presence of ergosterol in grass has not hitherto been fully established. We have obtained the characteristic absorption spectrum of ergosterol in the carefully purified total sterols isolated both from cocksfoot (kindly supplied by Prof. Chibnall) and perennial rye grass, the intensities corresponding with 1·0 and 1·4 % respectively. From this the ergosterol content of dried grass is of the order 0·002 %.

SUMMARY.

1. An experiment carried out in the winter of 1931–32 with four groups of cows was designed to measure the effects of two types of artificially dried grass, and of grass silage, on the quality of the resulting milk and butter in comparison with that produced on a normal winter ration. The values for the carotene, xanthophyll and vitamin A contents of the butters have been determined spectroscopically for the whole period of the experiment.

2. The results show that for cows fed on normal winter rations of hay and concentrates, the carotene, xanthophyll and vitamin A values fall steadily during the winter and remain at a very low level until the cows go out to grass in the spring. The consumption of fresh grass then results in a very rapid rise of the quantities of all three substances present in butter.

3. In the case of cows fed on artificially dried non-nitrogen-treated grass, the butters showed an increase in the content of the two carotenoids and of vitamin A very soon after the change over from the control diet. This higher level was then maintained until all the groups were again placed on the control diet, when the values dropped to the low control level. The results with the nitrogen-treated artificially dried grass were even better than with the non-nitrogen grass.

4. Grass silage of moderate quality was found to be little superior to the normal winter ration in its effect on the colour and vitamin A of the butter.

5. It may be concluded that the relatively high proportions of carotene, xanthophyll and vitamin A present in summer butter can be maintained during the winter period of stall feeding by the use of artificially dried grass.

6. In an examination of the unsaponifiable matter of grass it has been found that the spectroscopic blue test with antimony trichloride gives higher values for both carotene and xanthophyll than does the direct absorption spectrum method. The discrepancies are being investigated further.

7. The presence of ergosterol has been definitely established in the unsaponifiable matter of grass.

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CXV. SOME FURTHER OBSERVATIONS ON FACTORS WHICH INFLUENCE THE COMPONENT FATTY ACIDS OF BUTTER.

By HARRY KENNETH DEAN
and THOMAS PERCY HILDITCH.

From the Department of Industrial Chemistry, University of Liverpool.

(*Received May 2nd, 1933.*)

Attention was drawn in a former communication [Hilditch and Sleightholme, 1930] to the somewhat abrupt increase in iodine value and diminution in Reichert-Meissl value of butter-fats from cows when the latter had returned from winter conditions to spring pastures; the detailed analyses then available showed, correspondingly, that the butter-fat from cows on spring pasture contained considerably more oleic acid and somewhat less butyric, caproic and stearic acids than that from the same animals a few months earlier (when they were stall-fed).

The animals upon which these observations were based were from the typical herd at the National Institute for Research in Dairying, Shinfield. We have since been supplied with further samples of butter-fat from the milk of members of the same herd at intervals during the seasons 1930–32 by the courtesy of Captain Golding, who kindly informed us that in the spring season of 1932 attention was being focused at the Institute on the changes which occur when the cows go out to grass and suggested that a favourable opportunity offered for further detailed analyses of the butter-fat component acids during the transition from indoor to pasture conditions. In the course of the work it was found that, apart from the seasonal change under particular observation, the component acids of all three of the 1932 fats studied contained only 22–23 % (wt.) of palmitic acid as compared with 26–29 % (wt.) in the case of milk-fats from members of the same herd in 1928–29. This led us to examine the palmitic acid contents of the butter-fats in the intervening years, and the present communication accordingly deals with (i) the increase in the unsaturated character of butter-fat following the removal of the cows from stall to pasture in spring, and (ii) changes in the palmitic and oleic acid contents of milk-fats which are apparently connected rather with the age of the cows than with seasonal or similar influences.

The change in composition of butter-fatty acids following the transition of cows from stall to pasture feeding.

The iodine and Reichert-Meissl values of fats from the milk of cows from the typical herd taken at intervals from the autumn pasture season of 1928 to the early summer pasture conditions of 1932 establish clearly the presence of more unsaturated components in the butters from pasture-fed than stall-fed animals and, perhaps slightly less definitely, a correspondingly lower Reichert-Meissl figure, indicating somewhat less of the volatile (low molecular weight) fatty acids in the butters from pasture-fed cows. The butter-fats were not derived in every case from the milk of the same individuals, although these all belonged to the typical herd; in order to make the data more complete, therefore, it is convenient to refer to the individuals by letter and, in the first place, to give a list of the animals concerned (Table I).

Table I. *Cows supplying milk-fats dealt with in this paper.*

Reference letter		Date born	Date of first calf	Number of calves to May 1929	Number of calves to May 1932
A	Doll II	5. xi. 21	3. x. 24	5	7
B	Flora I	28. xi. 21	19. xi. 24	5	8
C	Pearl	17. iii. 23	28. xi. 25	4	6
D	Rosebud	3. xii. 23	4. xii. 26	3	6
E	White Rose	4. i. 24	29. viii. 26	3	4
F	Winsome III	14. x. 24	3. x. 27	2	5
G	Lottie II	2. iii. 25	16. ii. 28	2	5*
H	Peach II	3. iv. 25	25. xii. 27	2	5
I	Firefly III	24. ix. 25	21. viii. 28	1	4
J	Lucy III	9. iv. 27	7. xi. 29	—	1†
K	Lottie IV	17. iii. 29	21. xii. 31	—	1

* Including one on May 27, 1932.
† Lucy III sold fat after first lactation.

The complete series of iodine values and Reichert-Meissl values which have been determined on winter and summer butter-fats from these cows between 1928 and 1932 are given in Table II.

Table II.

Milk collected	Conditions	Cows supplying the milk	Butter-fats: Iodine value	Butter-fats: R.-M. value
28–30. x. 28	Late autumn pasture + stall	A, D, E, G, H, I	41·3	29·6
12–15. iii. 29	Indoor winter feed	*	34·8	32·3
26–27. v. 29	Early summer pasture	A, B, C, D, E, F, G, H, I	41·6	25·7
20–21. xi. 29	Indoor winter feed	B, E, I	42·2	27·0
18–19. xii. 29	,,	B, D, E, I, J	38·3	29·3
4–5. ii. 30	,,	B, D, E, I	38·9	27·3
24–25. ii. 30	,,	B, C, D, E, I	36·5	29·7
14–15. iii. 30	,,	B, C, D, E, F, I	37·3	30·6
25–26. iv. 30	Spring pasture	B, C, D, E, F, I	43·3	28·4
2–3. vi. 30	Early summer pasture	B, C, D, E, F, I	44·2	24·6
26–27. iii. 31	Indoor winter feed	F	38·9	—
,,	,,	B	37·7	—
10–11. v. 31	Spring pasture	F	44·6	—
,,	,,	B	44·4	—
,,	,,	D	40·7	—
21–22. iii. 32	Indoor winter feed	B, D, F, I, K	40·5	29·6
30. iv. and 1. v. 32	Stall + early spring pasture	B, D, F, I, K	45·9	25·7
27–28. v. 32	Early summer pasture	B, D, F, I, K	45·0	24·7

* Special feed including soya-bean cake, to 2 cows from the Shinfield herd not included in the list in Table I.

This group of general characteristics (for unsaturation and volatile fatty acid content) covers milk-fats taken over a considerable length of time from animals from the same herd and to a large extent from the same individuals. It is therefore probably safe to draw the following conclusions from the observations.

(1) The proportion of unsaturated acids is higher in the milk-fats of pasture-fed cattle than in those of the same cows under indoor winter conditions. The seasonal rise in iodine value of the fats is rather abrupt, the change being completed within 2 or 3 weeks (or perhaps even less) after the cows have been put out to grass.

(2) At the same time, there is a definite tendency for the iodine values of the pasture milk-fats to rise slightly but consistently throughout the series. Since the greater part of each sample included composite milk-fats from animals born in 1921 (B), 1923 (C, D), 1924 (E, F) and 1925 (I), there is reason to think that this slow increase in the maximum iodine values in succeeding years is a function of the age of the animal or (what is much the same thing) of the number of calves borne.

(3) The minima in iodine values for the winter milk-fats do not form so regular a series. Those for the winters 1929–30 and 1931–32 are 4–5 units below the preceding summer maxima, whereas those for the winters 1928–29 and 1930–31 show corresponding differences of nearly 7 units. It is probably significant that the smaller differences correspond with very mild winters and the larger ones with the much colder conditions of the early months of 1929 and 1931, a relation which was, indeed, pointed out in the previous communication [1930]. The degree of unsaturation of the winter fats may well be governed by at least three factors, namely (*a*) an increase in saturation, relative to that of summer pasture milk-fats, caused by either the indoor life or the indoor diet (or both), (*b*) the slow increase in unsaturated acid content suggested by the outdoor data to be connected with increasing age of the animals, and (*c*) a seasonal variation dependent upon the average temperature of a particular winter.

(4) Throughout the series the Reichert-Meissl values of the milk-fats tend to vary inversely with their iodine values, but they do not alter so regularly and abruptly as the latter. Moreover, it appears from the early spring pasture samples of April 1930 and April–May 1932 that the characteristic, relatively high iodine value of the pasture fats has already been attained, but the Reichert-Meissl value is intermediate between the higher values for milk-fats of winter, stall-fed cattle and the lower values finally reached when the cows have been at pasture for some time.

The foregoing review, based upon the average characteristics as defined by iodine and Reichert-Meissl values, has been supplemented by full analyses of the component acids present in the butter-fats of March, April and May, 1932. As already mentioned, Hilditch and Sleightholme [1930] pointed out apparent differences in the proportions of some of the individual fatty acids from analyses of the mixed fatty acids of autumn pasture 1928, winter feed 1929 and early summer pasture 1929 butters. The data for the winter-fed sample were primarily collected, however, in an attempt to study the effect of added soya-bean and coconut oil-cakes in the diet, and the simple winter-summer variation in composition was thus to some extent obscured; also, the two cows used for the soya-bean cake experiment were unfortunately not amongst those listed in Table I, from some or all of which all the remaining milk-fats dealt with in Table II emanated. In the present instance, 4 of the 5 cows concerned had been used frequently in providing specimens earlier in the series, and the stall diet in the winter of 1931–32 (*cf.* Table III) was normal and contained no special additions of fatty matter. The fresh detailed analyses therefore bear more directly on the point under discussion, although, as already stated, the difference in character (*e.g.* iodine values 40·5 and 45·9) of the 1932 stall-fed and pasture butters is unfortunately not so extreme as in some of the former years.

Full details of the cows contributing the composite samples of butter studied, and of their daily rations throughout the period concerned, are given in Table III.

Table III. *Details of cows used for production of butter-fat in winter to summer change of food, 1932.*

		Individual cows used for composite sample						
		Flora I	Rosebud	Winsome III	Firefly III	Lottie IV		
Date of birth of cow		28. xi. 21	3. xii. 23	14. x. 24	24. ix. 25	17. iii. 29		
No. of calves before 20. iii. 32		8	6	5	4	1		
Date of last calving		7. xi. 31	8. i. 32	20. ii. 32	12. i. 32	21. xii. 31		
		lbs.	lbs.	lbs.	lbs.	lbs.	Total lbs. milk	Fat* %
Milk yields	21. iii. 32 p.m.	21	20	19	12¾	11	83¾	4·10
	22. iii. 32 a.m.	27	27½	29½	22¼	18½	124¾	3·25
	30. iv. 32 p.m.	19	20	21	13½	11	84½	4·78
	1. v. 32 a.m.	27¼	29	30	22½	19½	128¼	3·05
	27. v. 32 p.m.	21	16	22	12½	10½	82	4·3
	28. v. 32 a.m.	29	23	30½	19	18¾	120¼	2·88
Daily rations:								
Winter period up to April 25th	Meadow hay	14	14	14	14	14		
	Mangels (Red Intermediates)	50	50	50	50	50		
	Concentrate mixture†	21	21	18	15	14		
Intermediate period April 26th to May 1st	Hay	7	7	7	7	7		
	Mangels	25	25	25	25	25		
	Concentrate mixture	14	15	15	9	9		
	Grass	8.30 a.m. to 3 p.m.						
Summer period May 3rd onwards	Grass	Day and night						
	Concentrate mixture	11	11	12	7	6		

* These fat percentages were on the composite sample from the whole typical herd and not of the 5 cows.

† Concentrate mixture: Dried grains 2 parts; Maize germ meal 2 parts; Soya bean meal 1 part.

Prior to investigation the butters were melted, settled and decanted and filtered through paper in a steam-oven. Their general analytical characteristics were as follows.

Table IV.

Butter-fat from milk of	21–22. iii. 32	30. iv. and 1. v. 32	27–28. v. 32
Sap. equiv.	248·8	255·4	254·8
Iodine value	40·5	45·9	45·0
Reichert-Meissl value	29·6	25·7	24·7
Polenske value	1·7	1·5	1·5
Kirschner value	24·0	20·7	20·4
Refractive index $n_D^{60°}$	1·4473	1·4485	1·4482

The determination of component fatty acids was carried out as described in former communications [Hilditch and Sleightholme, 1930; Hilditch and Jones, 1929] and, since the full fractionation and analytical details are extremely lengthy, only the final summaries of the data for each group of acids (steam-volatile, "solid" and "liquid" non-volatile in steam) are given here (Table V):

Table V. *Summarised experimental data for component fatty acids of the 1932 butter-fats.*

		Acids non-volatile in steam			% (wt.)	% (mol.)
Acid	Volatile acids g.	Solid acids S g.	Liquid acids L g.	Total	(Excluding unsaponifiable matter)	
Butter-fat from milk of 21–22. iii. 32 (indoor, winter ration).						
	25·92	200·6	213·4	439·92		
Butyric	17·19	—	—	17·19	3·9	10·6
Caproic	6·72	—	—	6·72	1·5	3·2
Caprylic	0·75	—	2·42	3·17	0·7	1·2
Capric	0·93	—	7·42	8·35	1·9	2·6
Lauric	—	1·05	15·31	16·36	3·7	4·4
Myristic	—	18·77	18·20	36·97	8·4	8·8
Palmitic	—	96·30	0·17	96·47	22·0	20·5
Stearic	—	65·65	—	65·65	15·0	12·6
Arachidic	—	2·93	—	2·93	0·7	0·5
Oleic	0·33	15·80	152·66	168·79	38·5	32·5
Linoleic	—	—	16·02	16·02	3·7	3·1
Unsaponifiable	—	0·10	1·20	1·30	—	—
Butter-fat from milk of 30. iv. and 1. v. 32 (intermediate period).						
	23·53	186·0	227·0	436·53		
Butyric	14·13	—	—	14·13	3·3	8·9
Caproic	7·31	—	—	7·31	1·7	3·5
Caprylic	0·73	—	2·32	3·05	0·7	1·2
Capric	1·11	—	6·89	8·00	1·8	2·6
Lauric	—	—	9·86	9·86	2·3	2·7
Myristic	—	15·73	22·72	38·45	8·8	9·4
Palmitic	—	92·16	2·90	95·06	21·8	20·6
Stearic	—	55·05	—	55·05	12·7	10·8
Arachidic	—	1·82	—	1·82	0·4	0·3
Oleic	0·25	21·14	155·89	177·28	40·7	35·0
Linoleic	—	—	25·19	25·19	5·8	5·0
Unsaponifiable	—	0·10	1·23	1·33	—	—
Butter-fat from milk of 27–28. v. 32 (early summer pasture).						
	23·09	182·8	225·2	431·09		
Butyric	13·23	—	—	13·23	3·1	8·5
Caproic	7·21	—	—	7·21	1·7	3·5
Caprylic	1·56	—	1·52	3·08	0·7	1·2
Capric	0·67	—	7·22	7·89	1·8	2·6
Lauric	—	—	13·56	13·56	3·2	3·8
Myristic	—	12·87	17·51	30·38	7·1	7·5
Palmitic	—	93·66	4·32	97·98	22·8	21·6
Stearic	—	53·60	—	53·60	12·5	10·7
Arachidic	—	3·17	—	3·17	0·7	0·6
Oleic	0·42	19·36	157·95	177·73	41·3	35·6
Linoleic	—	—	21·92	21·92	5·1	4·4
Unsaponifiable	—	0·14	1·20	1·34	—	—

We shall, as usual, discuss these figures mainly from the point of view of the molar distribution of the fatty acids present, and, for convenience, these are placed side by side in Table VI, together with the molar amounts of the following groups of acids: butyric-lauric, myristic-palmitic, C_{18} acids, oleic-linoleic [*cf.* Hilditch and Sleightholme, 1931].

Table VI. *Molar distribution of the component fatty acids in the 1932 butter-fats.*

Fat from milk of Acid	21–22. iii. 32 (Indoor, winter ration)	30. iv. and 1. v. 32 (Intermediate period)	27–28. v. 32 (Early summer pasture)
Butyric	10·6	8·9	8·5
Caproic	3·2	3·5	3·5
Caprylic	1·2	1·2	1·2
Capric	2·6	2·6	2·6
Lauric	4·4	2·7	3·8
Myristic	8·8	9·4	7·5
Palmitic	20·5	20·6	21·6
Stearic	12·6	10·8	10·7
Arachidic	0·5	0·3	0·6
Oleic	32·5	35·0	35·6
Linoleic	3·1	5·0	4·4
Butyric-lauric	22·0	18·9	19·6
Myristic-palmitic	29·3	30·0	29·1
C_{18} (+arachidic)	48·7	51·1	51·3
Oleic-linoleic	35·6	40·0	40·0

The fats from the "intermediate period" and the early summer pasture were closely similar, judged by their general characteristics (Table IV), and this is borne out to a remarkable extent by the component fatty acid figures in Table VI. Clearly, an increase of about 4 (mols.) % has occurred in the amount of unsaturated acids as a result of the change from indoor to pasture conditions, whilst the palmitic acid content has perhaps also increased slightly. The compensating fall in molar amount of saturated acids appears to be confined mainly, and in about equal proportions, to butyric and stearic acids; the individual figures for the other acids show no significant change (although those for lauric and myristic acids are less regular than the rest). In these complex mixtures, of course, too much reliance must not be placed on fractional percentages; but we believe that the close accordance between the observed compositions for the second and third samples justifies the general conclusions which have just been given.

The totals of the molar contents of butyric-lauric acids, the "volatile" acid group, range from 18·9 to 22·0 % in all three butters, and thus remain almost within the limits suggested by Hilditch and Sleightholme [1931] as characteristic for normal butters: "this figure appears to tend to a constant value of about 21–22, four of the six fats giving values between 20·7 and 21·6" (p. 518). The corresponding totals for the myristic-palmitic and C_{18} groups are, however, distinctly different from those given previously (C_{14}–C_{16}, 29–30 instead of 34–36, and C_{18} group, 48·7–51·1 instead of 43–44); comparison of the individual component acids of Hilditch and Sleightholme's Shinfield pasture-fed butters [Nos. I and IV, 1930] with the present series will show that this difference is mainly accounted for by a general fall of several mols. % in palmitic acid and an increase in both stearic and unsaturated acids. This change appears to be unconnected with the seasonal alteration in the composition of the milk-fat and to be a function of the age or progressive development of the cows concerned (*cf.* below).

Progressive changes in the composition of butter-fats apparently connected with increasing age of the cow.

We believe that the data presented in this series of observations show that, over a period of years, the milk-fat of a given cow tends to increase in its content of C_{18} acids and to diminish in that of palmitic acid. We have been able to obtain additional evidence on this point by a partial analysis of samples of 1930 and 1931 butter-fats from members of the Shinfield herd sent to us at those dates for determination of their general characteristics. Since in these cases a detailed component fatty acid analysis was not made at the time of receipt, and in view of the fact that (owing to the onset of slight rancidity) determination of the volatile and of the unsaturated acids at the time the present experiments were undertaken could not be relied upon to yield trustworthy results, a modified form of analysis only was made of the 1930–31 samples for the present purpose. The mixed acids from a known weight of butter-fat were at once submitted to the lead salt-alcohol separation (from which our customary addition of 0·5–1 % of acetic acid was omitted, since this procedure, which leads to more complete removal of oleic acid from the precipitated lead salts, also tends to take slight amounts of palmitic acid into the "liquid" acids); the "solid" acids obtained after recrystallisation of the deposited lead salts were converted into methyl esters and fractionally distilled in the usual manner. Using the modified lead salt method indicated, there is little chance of any measurable amount of palmitic acid escaping as dissolved lead salt, and it was thus possible to determine the palmitic and stearic acid contents of the 1930 and 1931 specimens.

In order to eliminate as far as possible any seasonal factors, the present comparison is based only upon the fats from pasture-fed animals of the seasons 1928–32 (in all but the first-named, the specimens were from early summer pasture feeding of about the same date in each case). Since it was only possible to obtain proportions by weight in the partially-analysed fats of 1930 and 1931, we must revert here to the somewhat less satisfactory comparison by weight, instead of by molar, percentages. The complete series of data is given in Table VII.

Table VII. *Weight-percentages of palmitic, stearic, oleic and linoleic acids in butter-fats from pasture-feeding, 1928–32.*

				Component fatty acids % (wt.)			
Milk collected	Pasture-feeding	Cows used	Av. age (years)	Palmitic	Stearic	Oleic	Linoleic
28–30. x. 28	Autumn	A, D, E, G, H, I	4·5	29·0	7·6	40·1	3·6
26–27. v. 29	Early summer	A, B, C, D, E, F, G, H, I	5·4	26·1	6·5	40·9	4·1
2–3. vi. 30	,,	B, C, D, E, F, I	6·5	23·6	8·3	Not determined	
10–11. v. 31	Spring	B	9·5	24·4	7·5	,,	
10–11. v. 31	,,	F	6·6	21·8	7·6	,,	
27–28. v. 32	Early summer	B, D, F, I, K	7·3	22·8	12·5	41·3	5·1

The cows contributing to the 1928 specimen were respectively seven (1), five (2), and three (3) years old; the 1929 sample included milk-fats from the same six animals and from three others then aged approximately eight (1), six (1) and five (1) years; whilst in 1932 four of the cows were amongst those used in 1928–29, but the fifth was a younger animal, then aged three years (the latter, however, only contributed about 14 % of the milk used to prepare the butter, 86 % coming from the four older animals). On the whole, and bearing in mind that it has not been possible to study the milk-fat produced by a single individual throughout the whole period of years, it seems fairly certain that the proportion of palmitic acid present declines with the age of the cows.

Certain well-defined similarities have been shown to exist [Hilditch and Sleightholme, 1931] between the milk and depôt fats of the cow, including not only a somewhat similar ratio in the amounts of palmitic as compared with the sum of the remaining acids, but also in some features of their glyceride structure, notably the relationship between the proportion of fully-saturated glycerides and of total saturated acids in the whole fats and a tendency towards identity in composition of the fully-saturated components within each type of fat. It may therefore be of some significance that Banks and Hilditch [1931] observed two classes of beef tallows, in one of which the molar content of C_{18} acids was about 68–70 %, while in the other it was about 61–63 %. The remaining acids, in this case, are palmitic with small amounts of myristic, so that these range from about 30 to 39 % (mols.); correspondingly, the weight-percentages of palmitic acid in the mixed fatty acids of the beef tallows examined by these authors and also by Armstrong and Allan [1924] vary over the range 25 to 33 %. In the light of the present results on milk-fats, it seems quite possible that the variation in palmitic acid content of beef tallows may, at least in part, depend upon the age of the animals when slaughtered. (In all the analyses referred to, the specimens were market tallows and there is thus unfortunately no information available as to the age of the animals which furnished them.)

SUMMARY.

A series of butter-fats, from milk of various (and frequently the same) members of the typical herd at the National Institute for Research in Dairying, Shinfield, at intervals from 1928 to 1932, has been studied by means of the usual analytical characteristics, supplemented by several detailed analyses of the component fatty acids. The earlier data appeared in a former paper, and the complete set affords an opportunity for detecting two characteristic changes in the composition of the butter fatty acids.

One change is seasonal and consists in an abrupt increase in the proportion of oleic (and linoleic) acids, with a parallel diminution in butyric and stearic acids, which is shown to have occurred in each of the four spring seasons when the cows return to pasture from indoor winter conditions. The increase in unsaturated acids amounts to about 4 % (mols.), and detailed analyses made on butters of the 1932 spring season show that this takes place within a very short time of the cows' return to pasture; the compensating diminution in saturated acids appears to be shared almost wholly between butyric and stearic acids.

The other change, which is a function of the age of the cow and not seasonal in character, consists in a gradual augmentation of the amount of unsaturated components of the fat over the whole period of years, mainly at the expense of palmitic acid, which, in the samples under review, declined from 29 to 22–23 % (wt.) of the mixed butter fatty acids between 1928 and 1932; the average age of the cows contributing the butter was 4·5 years in 1928 and 7·3 years in 1932, and the figures quoted refer in all cases to butters from pasture-fed cows (usually late in May or in early June).

The similar range of variation observed by Banks and Hilditch [1931] in the respective palmitic and C_{18} acid contents of beef tallows may therefore, it is suggested, also be connected with the age of the animal from which the depôt fat is obtained.

We offer cordial thanks to Captain Golding and Miss Dearden of the National Institute for Research in Dairying for the care taken in selecting the samples of butter and for providing us with full details of the individual cows used in each case; and to the Department of Scientific and Industrial Research for a grant to one of us (H. K. D.) during part of the time in which this work was in progress.

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CXVI. VITAMIN A AND CAROTENE.

X. THE RELATIVE MINIMUM DOSES OF VITAMIN A AND CAROTENE.

By THOMAS MOORE.

From the Nutritional Laboratory, University of Cambridge and Medical Research Council.

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Recent research has demonstrated that the richest vitamin A concentrates obtainable react in the antimony trichloride test some 10–20 times more intensely than carotene. Moreover, since the colorimetric, spectroscopic and analytical data given by concentrates prepared from a variety of liver oils tend to approach constant values, there are strong grounds for assuming that the concentrates represent more or less pure vitamin A [Karrer, Morf and Schöpp, 1931, 1, 2; Moore, 1931, 1; Heilbron *et al.*, 1932].

With such knowledge at our command it becomes possible to enquire into the relative efficiency of utilisation of the two substances in the animal, as indicated by the relative minimum curative doses. Since large dietary excess of carotene may lead to the excretion of the pigment in the faeces [Moore, 1931, 2; Ahmad, 1931] the writer at one time suggested that the conversion of carotene might be inefficient even at levels of intake approaching the minimum dose, and predicted that vitamin A concentrates much more active than carotene should be obtainable. This possibility seemed to be supported by the statement of Karrer, Morf and Schöpp [1931, 1] that a concentrate from *Hippoglossus* liver, which possessed biological activity 10 times greater than that of carotene, had been prepared, and also by the claim of Heilbron *et al.* to have obtained growth responses with vitamin A concentrates at levels much below the reputed minimum dose of carotene.

A review of the literature, however, reveals that the minimum doses quoted by different workers both for vitamin A and carotene vary over a wide range. In the case of carotene the dose necessary to restore growth was originally given by Euler, Euler and Hellström [1928] as 5γ (0·005 mg.), but recently Polak and Stokvis [1931; 1932] and also Goldblatt and Barnett [1932] have obtained responses at levels down to $0{\cdot}5\gamma$. In the case of vitamin A Euler and Karrer [1931] first gave $0{\cdot}5\gamma$ as the minimum dose of a vitamin A concentrate (*Hippoglossus*), a result in fair agreement with preliminary work by the writer [Moore, 1932] in which rather less pure specimens of rat-, pig- and turbot-liver oil concentrates were found to have minimum doses of the order of 1–2γ. Karrer, Morf and Schöpp [1931, 1][1], however, referring to work done by Euler, gave $0{\cdot}5\gamma$ as the dosage necessary for normal (not minimum) growth, while in a later communication Euler [1932] has quoted the same work of Karrer, Morf and Schöpp as having given the dose of vitamin A concentrate necessary for normal growth as $0{\cdot}1\gamma$, and has advanced fresh evidence that another concentrate, particulars of which are not given, could restore good growth at $0{\cdot}3\gamma$. Even lower doses have been found to be effective by Heilbron *et al.* [1932]. In a limited number of experiments these workers have observed good growth on $0{\cdot}1\gamma$, with definite signs of improved condition on $0{\cdot}025\gamma$.

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[1] These authors originally gave the dose for normal growth as 5γ but this was subsequently corrected to $0{\cdot}5\gamma$.

While small intrinsic differences in the biological activities of specimens of carotene and vitamin A concentrates prepared in different laboratories must almost certainly exist, it seems impossible that the extent of such differences could account for the wide range of minimum doses reported by different workers. On the other hand it is well recognised that results obtained in the biological test may be influenced by several variable factors, including differences in (1) the basal diet, (2) the experimental rats, both in regard to the initial vitamin A reserve and general state of health, (3) the medium used for diluting the test dose, and (4) the methods adopted in measuring growth responses, and in defining "normal" and "minimum" growth. Under these circumstances it is obvious that any attempt to deduce a ratio comparing the activities of the two substances from published data collected at random must be almost meaningless. To reach a satisfactory conclusion it is plainly necessary to carry out experiments in which the two substances are given in parallel experiments to closely matched groups of rats. This procedure has already been adopted in preliminary work, as a result of which it was inferred that vitamin A must possess not more than twice the biological activity of the international standard specimen of carotene. In certain of these experiments, however, a complication arose through the partial deterioration of the test solutions of vitamin A during the period of dosing, while on the other hand the standard carotene cannot be regarded as of the highest attainable purity. For these reasons the results of the preliminary experiments will not be described in detail in the present communication, but attention will be confined to a final experiment which is not open to criticism on these grounds.

EXPERIMENTAL.

24 young rats, 20 albino and 4 from piebald stock, all bred under conditions ensuring reasonably low initial vitamin A reserves, were fed upon a diet consisting of Glaxo caseinogen AB 15 %, rice starch 80 %, salt mixture 5 %, dried yeast 10 % (additional), radiostol 1 drop per rat per week. At the end of the preliminary depletion period (49–59 days) the rats were carefully paired for dosing either with carotene or a vitamin A concentrate. Each rat (with the exception of 1 pair, Nos. 10 and 10 *a*) was matched with a litter-mate of the same sex, the degree of similarity of the preliminary weight curves being taken into account when alternative choices were available.

Carotene. The carotene used was a highly purified specimen of β-carotene (2000 y.u., 350 b.u. per mg.) made available through the kindness of Prof. Richard Kuhn, to whom my best thanks are due.

Vitamin A concentrate. The concentrate was prepared from turbot-liver oil by alcoholic saponification, followed by the removal of sterols by crystallisation and precipitation by digitonin. It gave 3500 b.u. per mg. (= Carr-Price value 65000) and had $E^{1\%}_{1\,\text{cm.}}$ 328 $m\mu = 950$. As a basis for calculating the probable amount of "pure" vitamin A present in this concentrate the intensity of the absorption band at 328 $m\mu$ has been chosen as being less liable to personal error in observation than the blue value. In describing the richest concentrate Carr and Jewell [1933] have given the value $E^{1\%}_{1\,\text{cm.}}$ 328 $m\mu = 1600$. Taking this value as a provisional maximum for "pure" vitamin A it is obvious that 1γ of pure vitamin A was present in $1{\cdot}68\gamma$ of the concentrate.

Dosing. Coconut oil (B.D.H.) was chosen as a diluent for the test doses on account both of the great stability of solutions of carotene and vitamin A in this oil and of the absence of interference by the oil with the antimony trichloride test [Norris and Church, 1930]. Two stock solutions of carotene containing 1γ and $0{\cdot}1\gamma$ per drop were made up by dissolving a weighed amount of the pigment in chloroform, adding a small amount of oil, evaporating off the chloroform on a water-bath under diminished pressure and finally diluting to the required concentration by adding more melted oil. These stock solutions were kept in wide-necked bottles and stored throughout the experiment in a refrigerator.

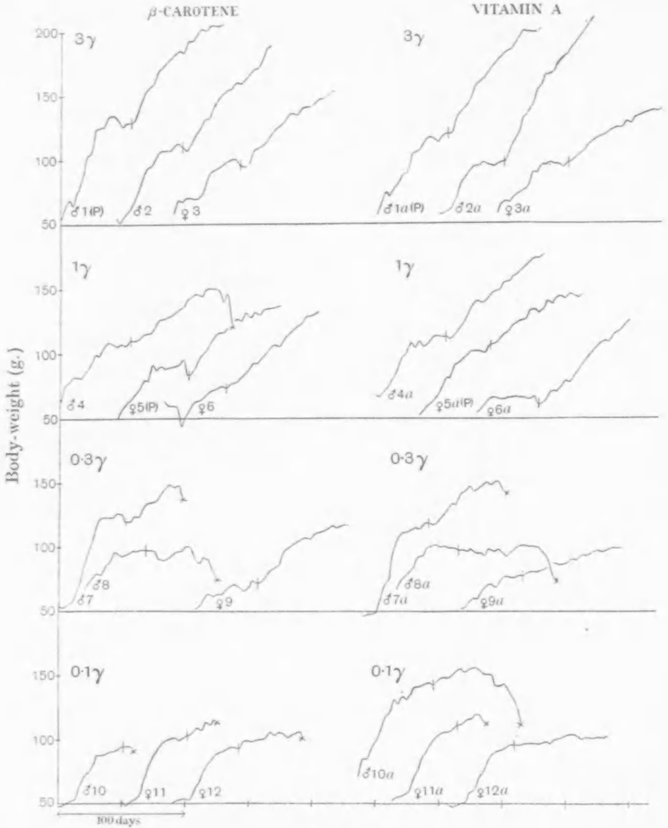

Fig. 1. Comparison of the biological activities of β-carotene (left-hand side of fig.) and vitamin A (right-hand side). The vitamin A was given as turbot-liver oil concentrate, $1{\cdot}68\gamma$ being assumed to contain 1γ of vitamin A. Paired rats are indicated by having the same number, the rat receiving vitamin A being in each case distinguished by the addition of the letter *a*. The rats having (P) after their numbers were from piebald stock, all the others were albinos.

Twice weekly amounts sufficient for immediate requirements were chipped off and melted in small conical flasks. Two similar stock solutions of the vitamin A concentrate, containing 1·68γ (1γ "pure" vitamin A) and 0·168γ were made up in the same way.

The stability of the test solutions was checked by observations of yellow value in the case of the carotene solutions, and of blue values in the case of the vitamin A solutions, the tests being applied to the portions of solution which had been in use for the preceding few days immediately before replacing by fresh solution from the stock bottles. No instances of deterioration were observed in the present experiments.

Dosing was carried out at the levels of 3γ, 1γ, 0·3γ and 0·1γ in the case of carotene, and at the corresponding levels of concentrate, 3 rats being used at each dosage. Treatment was continued for about 10 weeks, or until the previous death of the animal. The weight curves obtained are shown in Fig. 1.

DISCUSSION.

From the weight curves it will be seen that the responses caused by the corresponding doses of β-carotene and the vitamin A concentrate were very similar at all levels. Thus with 3γ of carotene or 3 × 1·68γ of the concentrate good growth resulted in every rat. At the 1γ level growth was less rapid, while one rat (4) receiving carotene declined and died suddenly after having grown steadily for 7 weeks. At the 0·3γ level the responses were irregular, but again the behaviour of paired rats was very similar. Thus rats 7 and 7 *a* showed a temporary restoration in growth followed by sudden decline and death, rats 8 and 8 *a* decline and death, rats 9 and 9 *a* slow growth. At the 0·1γ level all the rats died with the exception of 12 *a*, which survived in a weak condition for more than 10 weeks without showing much change in body weight, although xerophthalmia of gradual increasing severity persisted throughout the whole period.

It is plain, therefore, that β-carotene possesses biological activity equal to that of about the same weight of "pure" vitamin A, although the latter has a blue value some 10–20 times greater[1]. In other words the blue unit of carotene is equivalent to some 10–20 b.u. of vitamin A. If, moreover, we accept the data given by Carr and Jewell for their richest concentrate as applicable to pure vitamin A then it must follow that carotene is utilised in the body no less efficiently than preformed vitamin A, or at least that the difference in favour of vitamin A is only fractional, since the present experiments were not designed to detect any small difference in activity, such as might result from a loss of part of the carotene molecule during conversion.

The low minimum dose found for β-carotene raises another question which may deserve brief mention. Kuhn and his colleagues have reported that various other substances, *e.g.* α-carotene [Kuhn and Brockmann, 1931], γ-carotene [Kuhn and Brockmann, 1933], β-hydroxycarotene [Kuhn and Brockmann, 1932] and a new pigment isolated by Winterstein [1933] from the fruit of *Gonocaryum pyroforme* also possess biological activity. While no positive reasons for doubting these claims can be advanced the evidence put forward would be considerably strengthened if the biological activities were to be compared with that of β-carotene at levels approaching the minimum dose. This precaution would perhaps be most desirable in the case of Winterstein's pigment. This substance was separated by the Tswett absorption technique from a source which also contained α- and β-carotenes, in a yield so small (1·7 mg.) that extensive processes of purification could not be carried out. Since the dose given in biological tests was as much as 18γ it is obvious that some 5 % of β-carotene present as an impurity could account for the observed activity.

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[1] The exact ratio depends to some extent on the conditions under which the comparison is carried out. Thus the vitamin A blue tends to be diminished in the presence of oils, the carotene blue to be increased.

SUMMARY.

The biological activities of β-carotene and of a vitamin A concentrate were compared in parallel experiments. From calculations based on the amount of pure vitamin A presumed to be present in the concentrate it would appear that, contrary to views widely held, carotene is utilised in the body as efficiently as preformed vitamin A at levels approaching the minimum dose.

My thanks are due to Dr L. J. Harris for his helpful criticism, to Dr F. P. Bowden for carrying out a spectroscopic assay on the vitamin A concentrate and to Mr A. Davies for technical assistance.

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CXVII. THE GLYCOLYTIC MECHANISMS OF BRAIN.

By CHARLES AMOS ASHFORD.

(*Beit Memorial Research Fellow.*)

From the Biochemical Laboratory, Cambridge.

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In an earlier communication [Ashford and Holmes, 1929] evidence was brought forward of the existence in brain tissue of two glycolytic mechanisms, one concerned with the breakdown of glycogen, and requiring the presence of inorganic phosphate, the other with the breakdown of glucose and taking place without the participation of inorganic phosphate. This view of the non-participation of inorganic phosphate in glucose breakdown to lactic acid has received confirmation from later work by Bumm and Fehrenbach [1930; 1931]. They have studied the effect of the three factors, (i) *co-zymase*, which according to Meyerhof [1926, 1, 2; 1927] is concerned with the esterification and breakdown of the active phosphoric esters in the glycolytic mechanism of muscle, (ii) *hexokinase*, which Meyerhof [1927] had extracted from yeast, and shown to be concerned with an activation of glucose so that esterification, and breakdown of glucose took place in the presence of the muscle-enzymes, and (iii) the heat-labile *co-ferment T*, which they extracted from tumour tissue [Kraut and Bumm, 1928; 1929], and showed to accelerate the glucose breakdown by kidney, brain, white muscle and spleen, independently of the phosphate concentration. In the case of brain, Bumm and Fehrenbach [1931] showed that the rate of glucose breakdown was unaffected by addition of co-zymase, or by hexokinase, both of which are concerned with preliminary esterification of inorganic phosphate, but was increased by addition of co-ferment T. Furthermore, addition of arsenate, which is known to accelerate the rate of breakdown of phosphoric esters in muscle and yeast-juice, and hence to increase the velocity of carbohydrate breakdown, had no effect on the rate of glucose breakdown by brain tissue. Their experiments did not cover the glycogen mechanism, but they consider that their findings confirm our own with regard to the glucose mechanism in brain.

The experiments to be reported here are designed to amplify the evidence of the dual nature of the glycolytic mechanisms of brain tissue. There has not been entire agreement among workers as to a production of lactic acid from glycogen by brain tissue. Loebel [1925] did not consider that lactic acid was produced from glycogen, but he showed a production of small magnitude from hexosediphosphate. Tanko [1931] did not consider that his results showed a significant production even in the presence of added co-zymase. Haarmann [1932, 1] has results which indicate a production, on an average of the order of 24 mg. per 100 g. tissue in 3 hours, and this figure is in good agreement with those of Ashford and Holmes [1929] and with the figures reported here. Haarmann [1932, 1] also showed a production of 3 times that amount from hexosediphosphoric acid. From our point of view, a somewhat disconcerting observation is one of Haarmann and Stratmann [1932] that in the presence of glycogen, glucose breakdown is partially depressed, since this finding would not be in agreement with a theory of the independent nature of the two glycolytic mechanisms of brain. If such is the case the amount of glycolysis in the presence of both substrates should be equal to the sum of the amounts found when each substrate is present alone. Haarmann's results are rather puzzling, as the inhibition seems to be greater with increasing concentrations of glucose, and is actually greatest at concentrations of glucose which give maximum velocities of lactic acid production. If a common enzyme were involved, and it were a question of competitive inhibition by substrates, the contrary result would have been expected. His experiments have not been repeated in their entirety here, but no evidence of inhibition of glucose breakdown in the presence of glycogen has been detected, when the two are present in equivalent and maximum concentrations. On the contrary the observed production has never been less than the sum of the productions from the two substrates alone and in two experiments was practically equal to the sum of the two independent productions (see Table I). Further evidence on these lines is brought forward in this communication.

Table I. *Lactic acid productions from glucose, glycogen and glucose + glycogen.*

All values in mg. per 100 g. chopped brain tissue.

				Glucose + Glycogen	
Exp.	Time in hours	Glucose	Glycogen	Found	Calculated
1	3	443	33	626	476
2	3	406	34	540	442
3	2¼	262	11	296	273
4	3	380	27	417	417

Anaerobic incubation at 37° in phosphate buffer p_H 7·4.

General experimental methods.

In all experiments, 1 g. of chopped brain tissue of the rabbit has been used. This tissue has been weighed out into a pyrex boiling-tube, and the buffered Ringer solution p_H 7·4 (5 cc.) and the substrate (1 cc.) have been added. The tubes were closed by a rubber stopper carrying a bent glass tube and pressure tubing, which after evacuation was closed by a stout screw-clip. The tubes were evacuated at the water-pump for a period of 4 minutes and sometimes filled with nitrogen and re-evacuated, although the latter procedure is unnecessary with a good pump and was later abandoned. The tubes were fitted into a wire frame and connected to the shaking arm of a Barcroft thermostat at 37°. This shaking ensures more complete mixing of the tissue and substrate during anaerobiosis, and more consistent results are obtained if this procedure is followed than if the tubes are allowed to remain at rest in the thermostat during incubation. After the incubation period, the proteins were precipitated by addition of 10 cc. of 20 % trichloroacetic acid, and after standing for ½ to 1 hour, the mixture was filtered into a 50 cc. volumetric flask. The tissue was subsequently ground with further small quantities of 20 % trichloroacetic acid and filtered. The combined filtrate was made up to 50 cc., and 30 cc. were usually taken for the removal of sugar by the $CuSO_4$ and $Ca(OH)_2$ method. Lactic acid was then estimated on 5, 10 or 15 cc. of the filtrate by the aeration method of Friedemann, Cotonio and Shaffer [1927], blank estimations being regularly performed.

Experimental.

In the first place, an attempt was made to investigate the production of lactic acid in the presence of both glucose and glycogen. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. H_2O.
B. ,, ,, + 1 cc. 2·8 % glucose + 1 cc. H_2O.
C. ,, ,, + 1 cc. 2·8 % glycogen + 1 cc. H_2O.
D. ,, ,, + 1 cc. 2·8 % glycogen + 1 cc. 2·8 % glucose.

The p_H of the Ringer solution throughout these experiments was 7·4.

After incubation the protein precipitation and lactic acid estimations were carried out. The results of several experiments are shown in Table I. For the sake of clarity, only the actual lactic acid productions during the incubation period are given. In this and all subsequent tables the column labelled "calculated" is obtained by adding the observed productions from each substrate separately, *i.e.* the results obtained from tubes B and C. The first two experiments show, if anything, an increased production of lactic acid from glucose in the presence of added glycogen, and there is certainly no suggestion of inhibition of glucose breakdown. (At that time Haarmann and Stratmann's results were not to hand.) It should be noted that it was known from previous work that the concentrations of glucose and glycogen were sufficient to give a maximum and linear glycolytic rate over a period of 3 hours, and Haarmann and Stratmann agree with this in the case of glucose. Exps. 3 and 4 are in very good agreement with the theory that the lactic acid productions from glucose and glycogen proceed independently of one another, since the "calculated" and "found" values are in very good agreement with one another. It was felt, however, that owing to the fact that the lactic acid production from glycogen is quantitatively very small compared with that from glucose, it would be difficult to demonstrate accurately a summation of glycolysis in the simultaneous presence of both substrates, in view of the experimental error which is inherent in investigations of this type. It was known that considerably more lactic acid is produced from added hexosediphosphate than from glycogen (4 or 5 times, in fact), and, since the evidence was that a hexosephosphate stage was part of the glycogen mechanism, it was decided to try the result of competition between glucose and hexosediphosphate, and at a later stage, competition between glucose and hexosemonophosphate ("Robison ester"). The hexosediphosphate used was a solution of the potassium salt obtained by grinding the calcium salt (Messrs Baeyer's "Candiolin") with the calculated amount of potassium oxalate, and centrifuging the precipitated calcium oxalate. The solution was tested for freedom from oxalate ions. The solution was 7·9 %, *i.e.* equivalent to 2·8 % hexose. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. H_2O.
B. ,, ,, + 1 cc. 2·8 % glucose + 1 cc. H_2O.
C. ,, ,, + 1 cc. K hexosediphosphate + 1 cc. H_2O.
D. ,, ,, + 1 cc. K hexosediphosphate + 1 cc. 2·8 % glucose.

The results of several experiments are shown in Table II. It will be seen that in the main (in fact, in 5 out of the 6 experiments) the results found and the results calculated are in agreement with a theory of the dual nature of the modes of lactic acid formation. The average values of the "found" and "calculated" quantities are, in fact, in very good agreement with such a view.

To extend this idea further, the experiments were repeated using hexosemonophosphate instead of the diphosphate, since this substance probably

Table II. *Lactic acid productions from glucose, hexosediphosphate, and glucose + hexosediphosphate.*

All values in mg. per 100 g. chopped brain tissue.
Anaerobic incubation at 37° in phosphate buffer p_H 7·4.

Exp.	Time in hours	Glucose	Hexosedi-phosphate	Glucose + Hexosediphosphate	
				Found	Calculated
1	3	454	174	674	628
2	3½	493	108	605	601
3	3	453	147	487	600
4	3	284	157	469	441
5	3	327	167	473	494
6	3	340	162	507	502
			Average	536	544

approximates more closely to the "active" ester concerned in glycogen breakdown by muscle than does the diphosphate, if we accept Meyerhof's [1926, 2] view. The monophosphates used in these experiments were (i) a sample prepared by the action of maceration juice on glucose as described by Harden [1932], and was therefore a mixture of the glucose, mannose and fructose phosphates, and (ii) a sample of the barium salt obtained from British Drug Houses. The potassium salt was prepared by treatment with the calculated amount of potassium sulphate and was made equal to 5·1 %, *i.e.* equal to 2·8 % hexose. Tubes were set up as in the previous experiment except that the monophosphate took the place of the diphosphate. The results are shown in Table III, and it will be seen again that the quantities observed and those calculated are in very good

Table III. *Lactic acid productions from glucose, hexosemonophosphate, and glucose + hexosemonophosphate.*

All values in mg. per 100 g. chopped brain tissue.
Anaerobic incubation in phosphate buffer p_H 7·4.

Exp.	Time in hours	Glucose	Hexosemono-phosphate	Glucose + Hexosemonophosphate	
				Found	Calculated
1	2½	322	71	381	393
2	3½	493	109	618	602
3	3	453	91	352*	544*
4	3	284	92	311	376
5	3	300	97	432	397
6	3	338	82	396	420
7	3	419	110	513	519
			Average	458	449

* Omitted from average.

Table IV. *Lactic acid productions from different concentrations of hexosemono- and hexosedi-phosphates.*

Concentrations of substrate as in text.
All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° for 2 hours.

Amount of substrate added cc.	Hexosemono-phosphate	Hexosedi-phosphate
1	63	91
0·5	58	101
0·25	1	54

agreement with the theory that the two modes of breakdown involve different enzyme systems. It is further shown in Table IV that the concentrations of phosphoric esters employed were sufficient to maintain the maximum rate of glycolysis over a 2-hourly period. By comparison with Table V, where the results for a 3-hourly period are given, it will be seen that, at any rate up to 3 hours, the rate of lactic acid production is approximately linear.

Table V. *Lactic acid productions from different sugars by brain tissue.*

All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° for 3 hours. The concentration of substrate was 0·4 % or its equivalent.

Exp.	Glucose	Mannose	Fructose	Galactose	Hexosemono-phosphate	Hexosedi-phosphate
1	416	340	38	58	91	147
2	385	475	35	93	92	157
3	350	436	23	79	97	167
4	428	472	—	—	82	162
Averages	395	430	32	77	91	158

As a confirmatory point the effect of the addition of other hexoses to the brain + glucose was tried. The relative rates of glycolysis of other hexoses were first of all investigated, and the findings are shown in Table V. It will be noted that the lactic acid production is considerably less from fructose than Haarmann [1932, 1] gives, and is greater in the case of hexosediphosphate. For the former substrate Haarmann gives 90 mg./100 g. in 3 hours, and for the latter 76 mg./100 g. It is to be noted that both Kahlbaum and B.D.H. hexoses were used and gave the same result. It was quite obvious that mannose was the best choice for these experiments as it is glycolysed at a very similar rate to glucose. Tubes were therefore set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. water.
B. ,, ,, + 1 cc. 2·8 % glucose + 1 cc. water.
C. ,, ,, + 1 cc. 2·8 % mannose + 1 cc. water.
D. ,, ,, + 1 cc. 2·8 % mannose + 1 cc. 2·8 % glucose.

The results of four experiments are shown in Table VI. It will be seen that with the possible exception of Exp. 2, where, in any case, the lactic acid production from glucose is abnormally low, the lactic acid production in the presence of the two sugars together is never significantly greater than in the presence of glucose alone, and never in any case approaches the sum of the two ("calculated"

Table VI. *Lactic acid productions from glucose, mannose, and glucose + mannose.*

All values in mg. lactic acid per 100 g. tissue.
Anaerobic incubation at 37° in phosphate Ringer solution p_H 7·4.

				Glucose + Mannose	
Exp.	Time in hours	Glucose	Mannose	Found	Calculated
1	2	170	158	178	328
2	2	110*	140	148	250
3	2	287	253	277	540
4	2	240	295	317	535
	Averages	202	212	230	413

* Bad lactic estimations.

column). In this respect it differs markedly from the two cases of glucose + hexosemonophosphate, and of glucose + hexosediphosphate, and makes it reasonably certain that the same enzyme system is responsible for the breakdown of glucose and mannose. As a final point, the effect of the addition of both mono- and di-phosphates together was tried. Tubes were set up as follows:

A. 1 g. tissue + 5 cc. phosphate Ringer + 2 cc. water.
B. ,, ,, + 1 cc. hexosemonophosphate + 1 cc. water.
C. ,, ,, + 1 cc. hexosediphosphate + 1 cc. water.
D. ,, ,, + 1 cc. hexosediphosphate + 1 cc. hexosemonophosphate.

The results are shown in Table VII, and are a little surprising in so far that the observed value is never so large as the value calculated from the sum of the separate productions, but is consistently higher than the production from the most rapidly glycolysed substrate (the diphosphate). This effect cannot be due to the absence of sufficient substrate when one only is present, since it is shown (see Tables IV and V) that half the concentration of the substrates actually used will give a maximum velocity of glycolysis up to 2 hours, at least, and that in the concentrations employed in the experiments summarised in Table VII, the lactic acid production is linear up to 3 hours. We are therefore thrown back on the assumption that the mechanisms responsible are alike in many respects, but have at some point a different path. This point is briefly discussed later.

Table VII. *Lactic acid productions from hexosemonophosphate, hexosediphosphate, and hexosemonophosphate + hexosediphosphate.*

All values in mg. per 100 g. tissue.

Exp.	Time in hours	Hexosemono-phosphate	Hexosedi-phosphate	Monophosphate + Diphosphate	
				Found	Calculated
1	$3\frac{1}{2}$	109	108	193	217
2	3	91	147	152	238
3	3	92	157	180	269
4	3	97	151	201	248
	Averages	97	141	181·5	243

DISCUSSION.

The experiments described here amplify the evidence that brain tissue contains two mechanisms for the production of lactic acid. The limited ability of brain tissue to produce lactic acid from glycogen may, with a certain measure of probability, be regarded as being due to its relative inability to synthesise active phosphoric esters, since it is shown that hexosemono- and hexosedi-phosphates are converted into lactic acid to a much greater extent than is glycogen. This relative inability may be due to the low concentration of the "co-enzyme" factor, for, in the case of muscle, Meyerhof [1927] has shown that lactic acid is produced only from the phosphoric esters and not from glycogen in presence of a low concentration of the co-enzyme. It is now generally agreed that "co-zymase" consists of adenyl pyrophosphate, and that in the presence of magnesium ions [Lohmann, 1931], this substance will activate the "apozymase" of muscle. In the case of brain, Pohle [1929] has demonstrated the presence of adenylic acid and the author has shown the presence of pyrophosphate [Ashford and Holmes, 1929; 1931], while Sym, Nilsson and Euler [1931], from fermentation experiments with extracts of different organs, claim that brain, and more particularly grey matter, contains a fairly large amount of "co-zymase." On the other hand, Tanko [1931] was unable to demonstrate any significantly increased lactic acid production from glycogen by brain tissue, even in the presence of added co-zymase, so that in fact, the presence or absence of this factor is probably not the only reason for such findings. Nevertheless, the view has been taken in this work, that the systems responsible for the breakdown of glycogen and of the phosphoric esters in brain are almost entirely the same. The results shown in Table VII suggest that the mechanisms responsible for the breakdown of the hexosemono- and hexosedi-phosphoric esters follow some slightly different paths, but involve on the whole a common enzyme system. It is somewhat unexpected that the diphosphate is more easily broken down than the monophosphate, as the contrary result holds for muscle. It is unlikely that the mode of ester breakdown merely involves preliminary hydrolysis to the hexoses, since we have found that increased concentration of glucose above that used in these experiments does not increase the rate of breakdown. This fact was also shown by Haarmann and Stratmann [1932], using much larger amounts of tissue (2·5 g. instead of 1 g. in fact). Again, it is known from the work of Morgan and Robison [1928] that the hexosediphosphoric ester is constitutionally 2:5-fructose-1:6-diphosphate, and fructose has been shown to be very little attacked anaerobically by brain tissue. We do not of course know, however, how the unstable γ-fructose will behave in the presence of brain tissue.

Of the actual intermediary steps of the lactic acid production in brain we are extremely ignorant, nor indeed is there much certain information in the case of muscle. Haarmann [1932, 2] in the case of the dog, and Holmes [1933] in the case of the frog, have shown that methylglyoxal is rapidly converted into lactic acid by brain tissue, as is also pyruvic acid to a much less extent [Haarmann, 1932, 3]. In the first case the production of lactic acid is of the order of 763 mg./100 g. tissue in 3 hours, while in the second case, Haarmann gives figures of 149 mg./100 g. and 253 mg./100 g. in 3 hours. There seems little doubt of the probable occurrence of methylglyoxal in some stage in brain glycolysis. Glycolysis, for instance, is inhibited by iodoacetic acid, which is known to inhibit glyoxalase activity, and Dakin and Dudley [1913] and Meyerhof [1925] have shown the presence of glyoxalase in brain tissue. Meyerhof also showed that the rate of lactic acid production from methylglyoxal is 2 to 3 times as great as the rate when glucose is the substrate, suggesting that the limiting factor is the ability of the tissue enzymes to produce methylglyoxal from the carbohydrate. Case and Cook [1931] showed that anaerobic glycolysis in muscle was accompanied by a production of pyruvic acid, and that conditions which favoured lactic acid production, also favoured pyruvic acid production. It is tempting to speculate whether the glucose mechanism in brain may not give rise to methylglyoxal as intermediary, while the glycogen mechanism gives rise to pyruvic acid. So far, it must be admitted, there is no real evidence of the intermediary steps in brain glycolysis. Further work is obviously required in this field.

SUMMARY.

1. Glucose breakdown to lactic acid by brain tissue is not inhibited by the presence of glycogen, and the figures indeed suggest that the two mechanisms are quite independent.

2. When glucose and hexosemonophosphate or hexosediphosphate are present together, the glycolysis observed is the sum of the glycolyses produced from each substrate separately, showing that the enzyme systems responsible for the two effects are independent.

3. When glucose and mannose are present together, the results show that the same enzyme is responsible for their breakdown.

4. When hexosediphosphate and hexosemonophosphate are present together, the results suggest, in the main, a common enzyme system.

5. Figures are given for lactic acid production from glucose, mannose, fructose, galactose and the hexosemono- and hexosedi-phosphates by brain tissue.

It is a pleasure to thank Sir F. G. Hopkins for his interest in this work.

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CXVIII. THE DISTRIBUTION OF SULPHUR IN GOAT HAIR.

By DOROTHY JORDAN-LLOYD and
ROBERT HENRY MARRIOTT.

From the Laboratories of the British Leather Manufacturers' Research Association.

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During some recent experiments on the influence of various swelling agents on protein fibres it was noticed that, when treating hairs with concentrated solutions of sodium hydroxide in the cold, the cortex could be dissolved completely and the medulla, as far as microscopical appearance could tell, left intact. Human, horse and goat hairs could all be stripped of their cortex by this means, and it was therefore decided to make a sulphur analysis of the separated cortex and medulla. Although the absence of sulphur from the medulla is now generally accepted [King, 1933] and has been demonstrated by qualitative microchemical tests [Bekker and King, 1931] or inferred as a result of comparing data for medullated and non-medullated wool [Barritt and King, 1931], actual direct analysis of the two morphological parts of the hair shaft has not previously been recorded.

Goat hair was chosen as the material for the work since a large supply was available. The hair had previously been washed with a large quantity of clean tap-water and dried and degreased with acetone. A qualitative test on white hairs made on short cut pieces of the hair showed that, by soaking overnight in a solution of sodium cyanide and then adding sodium nitroprusside, a pink colour developed in the cortex while the medulla remained colourless. This suggested that the cystine group was mainly, if not exclusively, confined to the cortex.

A preliminary trial to select the most suitable concentration of sodium hydroxide for decorticating the hairs gave the following results (a large amount of reagent was used to a small amount of hair):

Concentration of NaOH solution	Condition after 5 hours	Condition after 24 hours
10 and 8 *N*	Cortex dissolved; medulla falling apart into discs	—
6·4 and 5·1 *N*	Hairs slightly coiled; cortex very swollen and medulla broken into segments	Cortex dissolved; medulla not in good condition
4·1 *N*	Hairs slightly coiled; cortex very swollen and medulla broken into segments	Cortex dissolved; medulla in good condition
3·3 *N*	Hairs violently coiled	Hairs just decorticated, medulla swollen
2·8 *N*	Hairs violently coiled	Hair in curly tangle which breaks on touching; cortex violently swollen; medulla broken
2·1 *N*	Hairs violently coiled	Cortex very swollen; bulging in places
1·7, 1·35 and 1·1 *N*	No change	Cortex swollen but intact

In order to effect the best separation of medulla and cortex while keeping down as much as possible the risk of damaging the medulla, $4N$ sodium hydroxide was taken. 11·31 g. of goat hair (containing 7·5 % of moisture) were mixed into 375 cc. $4N$ NaOH. A strong smell of ammonia developed. The mixture was left standing overnight at room temperature. The following morning a microscopic examination showed that the cortex had been completely dissolved while the medulla appeared to be intact. The medulla, buoyed up by air bubbles, was floating as a slippery mat at the surface of the solution. The containing vessel with its contents was, therefore, placed under a vacuum-pump to draw off the air bubbles. On returning to normal atmospheric pressure, the medulla residue left the surface of the liquid and became submerged. It did not settle to leave a clear, supernatant fluid. Solution and residue were separated by centrifuging. The supernatant solution was poured off, the residue washed twice with distilled water and separated each time by centrifuging. It was then washed with 50 % alcohol, centrifuged, washed with 70 % alcohol and again centrifuged. The residue now settled readily and completely in the wash fluids; it was washed twice with absolute alcohol and twice with ether. The residue was finally dried in the water-oven.

The dry weight of the medulla residue was 1·41 g. A portion was taken for nitrogen determination by Kjeldahl's method and a further portion for a sulphur determination by oxidation with bromine and fuming nitric acid and determination as barium sulphate. In carrying out the sulphur determinations the following method has been used.

When a strongly alkaline solution is under examination, it is advisable first to reduce the alkalinity, but to guard against loss of sulphur, should sulphides be present, by ensuring that the p_{H} of the solution does not fall below 10. Bromine water is added so that excess is present and fuming nitric acid is then added. Usually 5 cc. are sufficient. The liquor is evaporated slowly to dryness in a beaker over a water-bath and when thoroughly dry, a further addition of 5 cc. fuming nitric acid is made. A clock-glass is placed over the beaker and treatment on the water-bath continued until dryness is reached. In some cases, a further addition of nitric acid is required. Care is necessary to see that any residue left on the sides of the beaker comes in contact with the fuming acid. The dry residue is then treated with plenty of hot water, a small addition of hydrochloric acid made and after bringing to the boil the solution is filtered. The filtrate is brought again to the boil and the sulphur precipitated with barium chloride in the usual way.

The presence of a small quantity of flocculent material in the solution before filtering is of no moment as this, in our experience, is free from sulphur. The method is liable to error should barium compounds be present in the original solution. Should barium be present, filtering the solution after dissolving in water must be omitted, but the error will be very small if the original solution is free from insoluble inorganic material. A fairly large volume of water must be used and additions of hydrochloric acid are necessary should calcium be present so that the calcium sulphate is dissolved.

For solid materials, the protein should be moistened with bromine water and sufficient fuming nitric acid added to cover the material. The procedure then follows that given above.

The above method gives values for the total sulphur, which are reproducible, and which are sensibly the same as those obtained by the Denis-Benedict method. In our experience, the method is more reliable than this latter method and more suitable for fibrous substances.

The total volume of the alkaline solution of the cortex was taken as 375 cc. Nitrogen was determined on this fraction, after removing the free ammonia under reduced pressure at 40–45°. From this determination, the percentage of non-amide-nitrogen in the cortex was obtained and, after allowing 13·5 % as amide-nitrogen, the total nitrogen of the cortex was calculated. The total nitrogen of the original hair was also determined directly and from this figure, after allowing for the nitrogen of the medulla, the total nitrogen of the cortex was also calculated.

The following results were obtained:

Percentage composition of goat hair by weight:

Cortex	86·5	100
Medulla	13·5	

Sulphur content as percentage by weight:

Whole hair	3·0	By direct determination
	3·2	Calculated
Cortex	3·6	By direct determination
Medulla	0·23	By direct determination

Nitrogen content as percentage by weight:

Whole hair	17·3	By direct determination
Cortex	17·0	Calculated from nitrogen content of solution of cortex
	17·6	Calculated from nitrogen of original hair
Medulla	12·7	By direct determination
	15·3	Allowing for 13 % loss of N as amide

The small quantity of sulphur found in the medulla is almost certainly due to small traces of cortex left clinging to the medullary discs. It was difficult to give the medullary residue a thorough washing, partly on account of the strong alkali used and partly on account of the lengthy centrifuging required at the early stages.

The figure of 3·6 % for the content of sulphur in the cortex of goat hair is very close to that of 3·82 [Barritt and King, 1926] for non-medullated wool. King [1933] gives figures for merino wools (non-medullated) from 3·46 to 4·11 %.

A rough calculation (possibly a little premature) indicates the ratio of sulphur in keratin to the nitrogen of the backbone of the keratin molecule. This can be made by assigning 13·5 % of the total nitrogen to amide groups (Marriott [1931] gives 13·5 % for ox hair, a figure since confirmed; Van Slyke [1911] gives 10 % for dog hair, but ox is more nearly related to goat so the former figure has been preferred), and allowing for the guanidine groups of the arginine residues of keratin and the free amino-groups of the lysine residues. The yield of hair hydrolysates in arginine has been taken as 8 % and in lysine as 2 % approximately. This gives the following rough approximation:

100 g. cortex contain 17·6 g. nitrogen.
Amide-nitrogen = 2·4 g.
Guanidine-nitrogen = 1·9 g.
Free amino-nitrogen = 0·2 g.
Nitrogen from *R* groups[1] = 4·5 g.

Hence, nitrogen from backbone of the molecule = 13 g.
Sulphur can be taken as approximately 4 g.
Hence there is 1 S atom to every 7 N atoms from the backbone.

Astbury and Street [1931] have suggested that in natural keratin the backbone is thrown into folds and that the unit -N-C-C- belonging to each individual amino-acid residue is repeated in blocks of 3. One sulphur atom to every two blocks would, therefore, give an atomic ratio of N to S of 6 : 1. Since the 7 : 1 ratio obtained by calculation can only be looked on as exceedingly rough, it seems not impossible that, on the average, keratinisation leads to a protein formed by 1 cysteine residue to every 5 other amino-acid residues or 1 double residue of cystine to every 10 other residues.

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[1] "R" group indicates the residual portions of the amino-acids after allowing for the -N-C-C- portion which forms the "backbone" of the protein molecule.

SUMMARY.

1. The cortex can be separated from the medulla of goat hair by treatment with $4N$ NaOH.

2. The percentage content of sulphur in the separated cortex was 3·60 and in the medulla 0·23. It is suggested that the medulla is substantially free from sulphur.

3. It is suggested that keratin may contain 1 cysteine residue to 5 other amino-acid residues.

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CXIX. NOTE ON THE FORMALDEHYDE TITRATION OF MILK-PROTEIN, AND ITS APPLICATION TO THE ESTIMATION OF CASEINOGEN.

By GERALD THOMAS PYNE.

From the Department of Dairy Chemistry, University College, Cork.

(*Received May 8th, 1933.*)

The writer described in this Journal [1932] an improved technique for the determination of milk-proteins by formaldehyde titration. Very satisfactory results were obtained for mixed milks using a factor, 1 cc. *N* NaOH = 1·74 g. milk-protein, which however for milks of individual cows showed a somewhat wide variation, ranging from 1·63 to 1·87. It was tentatively suggested that this might arise from variations in the relative amounts of caseinogen and albumin from sample to sample and be susceptible perhaps of easy correction; and the further work now described has been carried out primarily to test this point. For this purpose the formaldehyde value, and protein content (N × 6·38) of a number of individual milks and of the corresponding caseinogen-free acid wheys were determined. Most of the samples were taken during two main periods—March to May 1932, and October to November, 1932—the entire herd of some 30 animals (Dairy Shorthorn breed) being tested on each occasion so as to obtain values representative of different stages of lactation.

The aldehyde values of the milks were obtained by the method referred to above and those of the acid whey somewhat similarly. The latter was prepared by a modification of the isoelectric precipitation method for caseinogen, recommended by Moir [1931], the principal changes being that the milk was not diluted with more than about 80 % of its volume of water before adding the acetic acid and sodium acetate solutions, and that these were employed in somewhat higher concentrations, *viz.* 2·5 *N* and *M* respectively. In this way too great dilution was avoided, the final volume before filtration not being greater than twice that of the original milk. For the determination of its aldehyde value 20 cc. of the filtrate (corresponding approximately to 10 cc. milk) was roughly neutralised with 2*N* NaOH, 0·4 cc. saturated aqueous potassium oxalate and 1·0 cc. 0·5 % phenolphthalein were added, and the liquid was accurately neutralised with *N*/10 NaOH, the end-point being a faint pink which was matched with that of a similar quantity of whey and oxalate tinted with magenta. 2 cc. 40 % formaldehyde were then run in and the titration continued to the same tint as before. Allowance must of course be made for the acidity of the formaldehyde under similar conditions of dilution, *etc.*

The results are summarised mainly as group averages in Table I.

The numbers in the column headed "g. caseinogen calc." were obtained by dividing the difference in the protein contents of the milks and their corresponding quantities of acid whey, by the difference in their respective aldehyde titrations, and are seen to display a marked uniformity over the whole period, the extreme variation in even individual samples being largely attributable to

Table I.

Months after calving	No. of samples	1 cc. N NaOH = g. milk-protein observed	g. whey-protein observed	g. caseinogen calculated	Ratio: $\frac{\text{Caseinogen-N}}{\text{Other N}}$
1	13	1·69	1·33	1·85	3·3
2	6	1·73	1·36	1·88	3·3
3	8	1·75	1·41	1·88	3·2
4	8	1·75	1·48	1·88	2·8
5, 6	3	1·76	1·49	1·88	2·9
7	3	1·76	1·54	1·84	3·1
8	5	1·77	1·52	1·87	2·7
9	3	1·78	1·58	1·85	3·1
10–13	5	1·80	1·59	1·89	2·8
Mean	(54)	1·74	1·44	1·87	—
Extreme values for individual samples		1·63–1·88	1·15–1·74	1·77–1·97	
Extreme variation from mean as % of mean		8·0 %	20·8 %	5·3 %	
Suggested % variation from mean attributable to experimental error		1·5 %	5·0 %	3·4 %	

experimental error. Omission of the correction for the volume of precipitated protein and fat does not appreciably affect the value in this column. The last line in Table I is based on the assumption that the titration errors in an average aldehyde value determination for total protein, whey-protein, and caseinogen (say: 2·0, 0·6, and 1·4 cc. respectively), do not exceed about 0·03, 0·03, and 0·05 cc. respectively (the allowance for the last is larger as the difference of two titrations is involved), and is included merely as a guide to the degree of constancy to be expected from, or shown by, the various factors.

From the data in Table I it may be seen that:

(*a*) The total protein value of 1 cc. N NaOH gradually increases throughout the lactation, and this increase does not appear to depend to any marked extent on variations in the ratio of caseinogen to total protein[1], but arises from the more strongly marked increase in the whey-protein value of the alkali during this period.

(*b*) The "caseinogen value" of 1 cc. N NaOH (mean 1·87 g.) is remarkably constant, making it evident that it is possible to estimate caseinogen by formaldehyde titration to at least the same degree of accuracy as total protein. If a 10 cc. pipette (delivering 10·25 g. milk), and $N/10$ alkali are used for the titration the formula becomes % caseinogen $= \frac{1{\cdot}87}{1{\cdot}025} \times$ (difference in formaldehyde values of 10 cc. milk and corresponding quantity of acid whey).

It seemed of interest to push the inquiry into variations in the formaldehyde factor of milk slightly further, and to look for the causes of the marked changes found in the whey-protein factor. The aldehyde titration of acid whey was already somewhat small for any detailed fractionation, and all that was attempted therefore was the removal of albumin by the usual heat-coagulation method and the determination of the nitrogen content and formaldehyde value of the filtrate. In six milk samples examined in this way, and chosen to represent various stages of lactation, the protein value of 1 cc. *N* NaOH was found to vary from 1·01 to 1·50 for the albumin-free filtrates, as against 1·22 to 1·47 for the corresponding acid wheys. While no great accuracy is claimed for these figures, on account of the smallness of the titrations on which they are based, they suggest that much of the variation in the formaldehyde factors of individual milks may be traced to variations in the composition of their non-protein-nitrogen fractions.

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[1] For about 95 % of the milk samples the ratio $\frac{\text{Caseinogen-N}}{\text{Other N}}$ lay between 2·5 and 3·8. The variation in the mean factor, 1 cc. N NaOH = 1·74 g. protein, arising from this cause would not exceed ±0·02, and is thus small compared with that found in practice for individual samples (see Table I).

SUMMARY.

1. The factor for converting formaldehyde titration into milk-protein shows a progressive increase throughout the lactation period, which appears to arise immediately from a similar but more marked increase in the whey-protein factor and to be ultimately attributable largely to variations in the composition of the non-protein-nitrogen.

2. The formaldehyde titration method can be adapted to the estimation of caseinogen.

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CXX. THE MINERAL CONTENT OF THE SKIN OF RATS SUFFERING FROM BERI-BERI, WITH INVESTIGATIONS ON THE FORMATION OF VITAMIN B_1 BY MOULDS.

By HUMPHREY THEODORE FAWNS and ALBERT JUNG.

From the Physiologisch-chemische Anstalt der Universität, Basle, Switzerland.

(*Received April 13th, 1933.*)

On the subject of mineral metabolism, and still more on that of the mineral content of individual organs, our knowledge still consists of a series of isolated facts. The summaries of our knowledge on this subject, made by Klinke [1931], Heubner [1931], Reichel and Spiro [1927], Höber [1924], Zondek [1927], Spiro *et al.* [1929], all show the fundamental connection between mineral metabolism and the physico-chemical processes in and around the cell and the effect of this on cellular activity and development. Furthermore, the internal secretions exert an influence directly and indirectly through general metabolism and nourishment.

Owing to the complexity of this relationship, it is readily understandable that the figures cited as "normal values" are liable to show wide variations, dependent on the previous history of the animal and the conditions obtaining prior to the withdrawal of the tissue sample. Hence, for investigating the factors controlling the mineral content of a tissue, values taken for comparative purposes must of necessity be obtained under the most comparable conditions possible. For instance, in investigations by one of us [Jung, 1931, 1], on tuberculous subjects, quite different results were obtained from specimens collected *in vivo* and from subjects who had died from tuberculosis. In the case of the latter, the long cachexia plays a rôle not to be underestimated. At the commencement of the investigation in hand, namely the effect of avitaminoses B_1 and B_4 (termed collectively beri-beri) on the skin of the rat, no figures which might serve as "normal values" were to be found in the literature, and the more recent work of Loewy and Cronheim [1932] plainly emphasises the importance of comparing only such values as have been obtained under conditions as nearly identical as possible.

Very little is yet known regarding mineral metabolism in rat beri-beri. The clinical symptoms (rough hide with moist patches—coldness—a bluish colour, and red, swollen extremities in avitaminosis B_4), together with the general metabolic disturbances, would lead one to expect changes in the mineral constituents of the skin. Furthermore in this disease, which results from a deficiency of a very minute constituent of the diet, it is possible that changes of the internal secretions play a large part (*e.g.* suprarenals, thyroid *etc.*), so that one cannot say with any certainty where the primary disturbance sets in. Moreover, the sharp loss in weight and deficient growth due to avitaminoses B_1 and B_4 must also be taken into consideration. Unfortunately it was not possible to carry out the necessary controls on as large a number of animals as one would have liked. Finally, this investigation was not designed to give the subject exhaustive treatment, but rather to determine as far as possible whether any specific conclusion could be drawn regarding the mineral content of the skin, which arose as a net result of these various complex influences. In carrying out these experiments, it was observed that a spontaneous formation of mould on the feeding cake appeared to inhibit the onset of beri-beri, and a further experiment was therefore conducted to elucidate this point.

VITAMIN B_1 FORMATION BY MOULDS.

In order to investigate the problem of whether moulds such as *Aspergillus niger*, which readily forms on the feeding cake, can produce this vitamin in sufficient amounts to satisfy rats and also to test whether the mould imparts the vitamin to the cake, and further, to ascertain in what part of the mould the vitamin is located, the following experiment was carried out. Ten animals were used, of which 5 at first only received vitamin B-deficient diet (BaW) until, after 27–44 days, they developed beri-beri. These animals were cured, with an immediate and rapid increase in weight, by the same basal diet on which *A. niger* had grown spontaneously. The animals still increased in weight well when the mould grew sparsely and for a time no blackening of the cake was obtainable. At this point, 3 animals were killed with ether for skin analysis. One of them, 2317, had received the mould-feeding for 26 days, the other two, for 56 and 50 days. The remaining animals showed a decrease in weight soon afterwards. This can be attributed on the one hand to scanty formation of the mould, and on the other hand to an overgrowth by a yellowish-green form of mould (*A. flavus*?). During this time, however, the cakes were always covered with mycelium. When for a few days no mould grew, the animals showed a sudden partial decrease in weight, but one of them, 2310, grew in spite of this. Renewed application of mould produced good growth once more. The animals developed unsatisfactory hides at this time, moulting and eczema formation occurring in places, which healed up after feeding with egg-white as the source of vitamin B_2, after the technique of Chick and Roscoe [1929]. The animals showed trembling of the head and heart-palpitations, were very nervously excited and had a severe thirst. This condition was cured by giving the animals the mould which had been cultured on rye-bread. This however did not produce the same rate of growth in the rats as that which grew on the basal diet; in fact one animal, 2308, even developed beri-beri. Although not studied quantitatively, in that the mould was only given in approximately equal quantities, this phase of the experiment seems to indicate that the mould when grown on rye-bread forms less vitamin B than when grown on the basal diet, for the animals' weights increased again as soon as they were given either the mould-covered basal diet or fresh basal diet + mould. That the mould yields little or no vitamin B to the cake itself is shown by results obtained during periods when only mould-cake was given from which the mould had been previously removed and which had been inactivated at 70° to prevent fresh conidia forming from the mycelium still present.

The sensitivity to lack of water was shown when, on one occasion, insufficient water was given. The animals lost 15–25 g. The success with which the rats thrive on the mould-covered food is shown by a young male that has now reached a weight of 219 g. after 236 days and is still growing. We are at present trying to use him as a breeding buck. The hide is still completely intact.

Five animals received the mould-covered basal diet throughout the experiment and grew well on it. In the case of 3 other animals it was only given after 49 days to one, and after 69 days to the other two. The growth of these animals ceased at once, they lost weight, and after 13–14 days beri-beri set in. One animal was successfully cured by mould feeding but this proved ineffective in another case. Three animals were taken for skin analysis.

It is intended to continue this investigation and grow the mould on artificial media, *e.g.* as described by Nielsen and Hartelius [1933], then once again on vitamin B-containing material such as wholemeal bread, but more especially, to carry out the work on a quantitative basis.

Up to the present it would appear that *A. niger* forms vitamin B chiefly in the conidia (spores), while the mycelium is relatively poor in it. It imparts very little vitamin B to the substrate. It seems to form less vitamin B when grown on rye-bread, and a yellowish-green mould (*A. flavus?*) appears to contain even less. There is evidence that not all the B-factors are formed in equal amounts (as judged by the symptoms; skin-exudate, derangements of balance, and trembling). The animals' severe thirst as well as their sensitivity to lack of water is quite remarkable.

In those groups of animals which had grown well, a very plentiful layer of fat occurred, especially in the lower part of the stomach and round the kidneys, similar to that described by Plimmer [1927] in relative vitamin B-deficiency. For the growth curves of these animals, see Fig. 1. The animals used in this experiment constitute groups 3 and 4.

The animals cited in Table III were used for skin analysis.

VITAMIN A CONTROL.

In order to be able to compare the results of the beri-beri experiments with the effect produced by vitamin A-deficiency, one animal, 2477, was taken, which, after 38 days of preparatory feeding, showed ophthalmic symptoms of vitamin A-deficiency.

The animal received the following basal diet: vitamin-free caseinogen (Wander), 18 %; coconut oil, 15 %; maize powder starch, 57 %; McCollum and Davis's salt mixture, 5 %; dry brewer's yeast, 5 %.

The diet was stirred into a paste with water, mixing 1 drop of "vigantol" with the fat per kg. diet, and the whole was dried at about 70° so as to form a cake. Shortly after the commencement of the experiment 0·5 g. of "Eimalzin" was administered. The ophthalmic conditions improved, with simultaneous increase in weight. Then the animal contracted a cold, producing a periodic bloody excretion, during the course of which rapid decrease of weight occurred, and the condition of the eyes got worse. Dyspnoea set in, the extremities became blue and the animal generally weak, while the excretion from the nose increased. It could only move shakily and on the last day could not even stand. The ophthalmic condition practically disappeared during the last few days.

Several symptomatic similarities to the B-avitaminosis appear in the clinical picture: *viz.* bluish extremities and derangement of balance. In this case the basal diet contained 5 % yeast, and 0·5 g. "Eimalzin" produced an increase of 50–60 g. in 35 days in the vitamin B experiment, on an average of 10 animals.

The vitamin A was at the lowest limit of its effectiveness, since out of 10 animals, only 5 produced an average growth of 29 g., the others did not increase in weight at all or else died.

EXPERIMENTAL.

Animal material. Rats of identical breed were used, which had all been fed on the same standard diet for several generations. The animals were initiated into the experiment at the age of 3–4 weeks. The following groups were investigated: Group 1, yeast controls; Group 2, vitamin B-deficient animals; Groups 3 and 4, the mould-fed animals cited above.

The animals received the following basal diet:

	BM %	BaM %
Caseinogen, Merck's vitamin-free ...	20	20
Maize powder starch	60	55
Coconut oil	10	10
Cod-liver oil...	5	5
Salt mixture, McCollum and Davis ...	5	5
Yeast, autoclaved	—	5

BaW diet is the same as BaM except that caseinogen of Dr Wander was substituted for caseinogen Merck. Both have similar biological values.

The dried brewer's yeast was autoclaved at a weakly alkaline reaction for 5 hours at 120°. As shown by test with the international standard product for vitamin B_1, 5 % of this autoclaved yeast is not sufficient as a source of the extra B-factor. 8 % is the minimum sufficient. In these experiments, this perhaps must be taken into account with the other groups which received no yeast as supplementary diet.

Animal 1485 increased in weight very little. Since it was also sharply differentiated from the others as regards the mineral content of its skin (high figures for Cl and P but low for Na), it was considered separately in the calculation of the average values.

Table I. *Group 1.* (*Yeast controls.*)

Animal No.	1485	1488	1507	1511	1497	1500	1502
Mother's No.	456	456	509	509	740	740	740
Sex	m	m	m	f	f	m	m
Colour	gw	w	w	w	sw	sw	sw
Basal diet	BM	BM	BM	BM	BaM	BaM	BaM
Supplement (yeast)	0·05	0·2	0·2	0·05	0·1	0·1	0·025
Initial weight (g.)	48	47	55	66	39	51	42
Final weight (g.)	74	128	149	109	139	155	136
Increase in weight (g.)	26	81	94	43	100	104	94
Duration of exp. (days)	57	57	57	57	57	57	57
Age at end of exp. (days)	110	110	101	101	96	96	96

m = male, f = female, gw = grey white, w = white, sw = black white.

Table II. *Group 2.* (*Beri-beri animals.*)

Animal No.	1482	1638	1721	2149	2157	2655
Mother's No.	1195	777	767	1581	1533	1232
Sex	f	f	m	f	f	f
Colour	w	w	sw	sw	w	w
Exp. commenced (1932)	30. iii.	11. iv.	20. iv.	9. vi.	9. vi.	15. vii.
Basal diet	BaM	BaW	BaW	BaW	BaW	BaW
Onset of beri-beri (days)	None	51	42	48	48	50
Initial weight (g.)	36	40	51	37	45	41
Maximum weight (g.)	49	58	57	48	58	55
Weight at death (g.)	32	43	40	33	38	39
Age at death (days)	99	85	67	70	78	73

Table III. *Mould-fed animals.*

	Group 3. Cured			Group 4. Beri-beri			Group 5 A-control
Animal No.	2312	2315	2317	2309	2311	2314	2477
Mother's No.	1236	1858	1858	750	1236	1858	1372
Sex	f	f	f	f	f	f	m
Colour	sw	sw	sw	sw	sw	sw	w
Basal diet	BaW	BaW	BaW	BaW	BaW	BaW	A-control
Exp. commenced (1932)	16·6	16·6	16·6	16·6	16·6	16·6	29·6
Onset of beri-beri (days)	33	40	44	13	34	37	(38-Ophth)
Age at death	110	110	90	84	110	110	112
Cause of death	E	E	E	sp	E	E	sp

E = Ether, sp = spontaneous, Ophth = ophthalmia, and remaining abbreviations the same as in Tables I and II. Weights are recorded in Fig. 1 below.

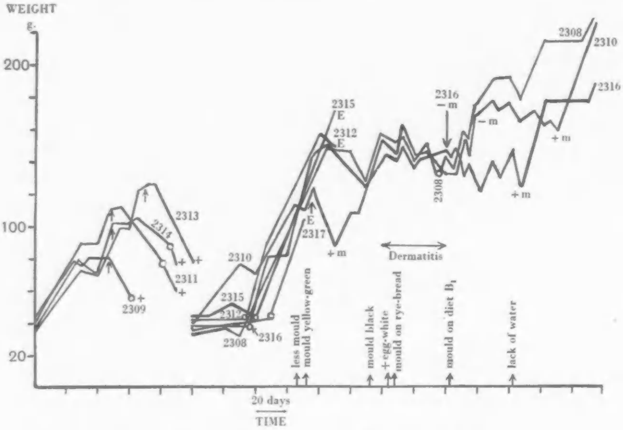

Fig. 1. Growth curves for mould-fed animals.

↑	food without mould.	E	killed with ether.
○	beri-beri.	+m	mould-diet with mould.
+	death.	−m	mould-diet without mould.

The dried brewer's yeast was obtained from "Vitamin Hefe, A.G.," of Rheinfelden, Switzerland. 0·05 g. gives a minimum increase of 50 g. in 5 weeks, 0·025 g. of about 30 g. (according to the usual vitamin B_1 test, with an average of 10 animals).

Whereas the Group 1 animals were all taken for the experiment on April 13th, 1932, the animals of Group 2 were commenced at varying periods and the initial dates are therefore inserted.

Animal 1482 was weak from the commencement. Its weight increased from 36 to 49 g. in the period from day 35 to day 52, then it decreased suddenly. The animal died on the 66th day without sign of beri-beri. The initial increase in weight is attributed to spontaneous mould formation, which, in common with similar previous observations, was the incentive for carrying out the experiment on vitamin B-formation by moulds, cited above.

Animal 2655 showed the usual symptoms of beri-beri on September 2nd. On the 3rd its hide was rough and moist, symptoms became more severe and death ensued on the 6th, or 4 days after onset of beri-beri cramp, whereas the remaining animals died at the most 1 day afterwards and showed beri-beri symptoms in even more pronounced forms.

The suprarenals of animal 1638 were weighed; they were, left, 6·94 mg.; right, 7·38 mg.; and from animal 1721 they weighed, left, 11·68 mg.; right, 10·48 mg.

Technique of skin analysis.

1. *Handling of the skin prior to analysis.* The skin was removed from the animal immediately after death, or when this was not possible, the animal was stored in the ice-chest until required. In any case, the removal of the skin took place within a few hours of the animal's decease.

The specimen of skin taken for analysis consisted of a complete band round the animal's body between the fore- and hind-legs, with the subcutaneous fatty layer adhering to it. The hair was removed with scissors and scalpels and the specimen immediately weighed out on to a watch-glass and dried in the oven at 100° to constant weight, this process providing also the estimation of moisture.

The dried specimen was then transferred quantitatively into an extraction cartridge and extracted with ether in a Soxhlet apparatus for 12 hours, previous preliminary estimations having shown that 12 hours was ample time for complete removal of the fatty constituents. The estimation of total ether-extractives is thus obtained and is set out in Table V (*a*), under the heading Fat. Only total ether-extractives were estimated; a detailed analysis of the fatty constituents was not undertaken. The first 3 specimens of ether-extract (Nos. 1638, 1721, 1482) were digested with nitric acid and the digest tested for chloride. No chloride was found.

The dried, fat-free skin was then weighed and transferred quantitatively into a quartz digestion flask, to which was added 10 cc. of $N/50$ silver nitrate and then 10 cc. fuming nitric acid. The digestion mixture was allowed to stand overnight and the digestion completed next day by boiling, a further quantity of nitric acid being added when necessary and finally 20 cc. of distilled water. The filtrate and washings were made up to 50 cc. According to Tschopp [1927], digestion in the presence of silver nitrate effects complete removal of the chloride by precipitation as silver chloride, which can then be determined by estimating the excess silver nitrate remaining in the solution. Therefore two portions of 10 cc. each were withdrawn from the 50 cc. ash solution and used for the chloride estimation in duplicate. The remaining 30 cc. was then treated with 1 cc. of $N/10$ HCl, to precipitate the excess silver remaining therein. 4 cc. of distilled water were then added, bringing the volume up to 35 cc. The silver chloride was removed by filtration.

The estimations of the remaining constituents were carried out in aliquot portions of this filtrate.

2. *Analytical methods.* The estimations were in most cases carried out in duplicate, the values, quoted in Table V, being the means of 2 estimations.

A negative control was also carried out, and the values obtained were deducted from the skin values where this proved necessary.

The methods of analysis selected were, in general, those of Rossenbeck [1930] and those employed in the more recently published work of Herrmansdorfer and Jung [1932–33], as follows.

(*a*) Chloride [Tschopp, 1927; also Rossenbeck, 1930; Herrmannsdorfer and Jung, 1932–33].

(*b*) Sodium [Blanchetière, 1923; also Hirsch, 1931].

(*c*) Potassium [Kramer and Tisdall, 1921, 1; also Rossenbeck, 1930; Herrmannsdorfer and Jung, 1932–33].

(*d*) Calcium [Kramer and Tisdall, 1921, 2].

(*e*) Total phosphorus [Bell and Doisy, 1920; also Rossenbeck, 1930; Herrmannsdorfer and Jung, 1932–33].

(*f*) Magnesium [Denis, 1922; also St Gadient, 1923; Herrmannsdorfer and Jung, 1932–33].

3. *Notes.* (*a*) It should be mentioned that in carrying out colorimetric phosphate estimations by the molybdan-blue reaction, which is necessary for determinations of sodium, magnesium, and total phosphorus, by the above-mentioned methods, "Eikonogen" was used as the reducing agent in place of quinol. This is the Na salt of 1:2:6-aminonaphtholsulphonic acid ("Agfa" product) and yields very satisfactory results [see also Hirsch, 1931].

(*b*) The values for total phosphorus do not include lipin P.

(*c*) The amount of fresh skin obtainable (less hair) varied from 1 to 7 g., according to the size of the animal, and correspondingly, the amount of dry fat-free skin available for ashing varied from about 0·3 to 2 g.

RESULTS.

Moisture contents. In Group 1 the moisture content of the skin together with the subcutaneous tissue varied between 42·7 and 56·8 %, in Group 2, from 58·5 to 68·4 %, and is therefore significantly higher. The same can be seen in Groups 3 and 4. The moisture content in the beri-beri and A-control animals is only noticeably raised on account of the corresponding disappearance of the fat. This reciprocal effect is also seen by a comparison of these values and those of Loewy and Cronheim [1932], in that their moisture content corresponds with our Group 2, and the fat content is similarly low.

Fat content. By fat is implied the substances which are soluble in ether. The loss of flesh is significantly shown in the lowered fat content of the skin. Whereas in the well grown Groups 1 and 3, the fat content varied from 16·8 to 31·1 %, in the case of the poorly grown animal 1485, it was only 14·9 %, and in the case of animals with more marked loss of weight, it had dropped to minimum values of 1·1 and 0·6 % in Groups 1 and 2 respectively, and only one animal, 2149, was able to show 6·4 %.

Although the mineral constituents are in part dissolved in the tissue fluid, and the calculation on a fresh-weight and a fat-containing dry-weight basis has its justifications, the calculation on a fat-free dry weight basis is preferable, on account of the variations of fat-content, at least in tissues relatively rich in fat. The possibility of some of the mineral constituents being removed with the fat must also be borne in mind.

Sodium content. The Na contents of the skins of our Group 1 are somewhat high by comparison with the normal animals of Loewy and Cronheim [1932], although the 2 animals cited by these investigators form hardly a sufficient basis for "normal values." Brown [1926] obtained for dogs, values of 155–250 mg./100 g. dry-weight, and for rabbits 116–243 mg./100 g., values which are somewhat lower than those for dogs obtained by Meyer-Bisch and Bock [1927], who found 115–145 mg./100 g. before pancreas extirpation and 87–156 mg./100 g. afterwards, as fresh-weight values. Our values of 85–104 mg./100 g. fresh-weight, correspond approximately with these figures, if one is justified in comparing values from different animal species. The Na content for human beings seems to be somewhat higher than for the animals investigated to date, as is shown by the analyses of Brown [1927] who found 118–188 mg./100 g. in the fresh tissue, and similar work by Jung [1931, 1] and Herrmannsdorfer and Jung [1932–33] in which 111–222 mg./100 g. fresh-weight was found.

If the Group 1 figures are taken as a basis, then the 2 poorly grown animals without beri-beri (Nos. 1485 and 1482) show a considerable decline, likewise the corresponding animals of Group 2, but without any significant changes seeming to be specific for beri-beri; this also applies to Group 4.

Potassium content. With this element important variations exist between the individual groups. Marked variations occur even within Group 1 itself, which also occurred in the work of Loewy and Cronheim [1932]. Our values lie approximately at the upper limit of the normal, both in comparison with their work and also with the work on mice by Börnstein [1926], who obtained values from 90 to 131 mg./100 g. for fresh cutis (similarities exist between the diet of these mice and that of our Group 1 animals). Stern [1932, 1], with guinea-pigs, obtained values of 351–590 mg./100 g. dry, fat-containing skin, and approximately 120–180 mg./100 g. fresh-weight (dry substance averaged 30 %), which was therefore of about the same order as our Group 1. It was not possible to ascertain the influence exerted by colour, sex or yeast administration (so far as that produced normal growth), except that the animals receiving autoclaved yeast in the basal diet showed somewhat higher results. This applies especially to No. 1502, in which, despite good growth the 0·025 g. yeast was perhaps not quite adequate.

The rise in K values in the other groups is very remarkable. No. 1482, which also had a low Na content and no beri-beri symptoms, forms an exception. The fact that for 2 animals of group 3 definitely raised values were found makes one consider that the time allowed to elapse during the investigation (*e.g.* only 26 days in the case of No. 2317) was perhaps not always sufficiently long to effect an appreciable change in the somewhat slow-acting mineral interchanges of the skin [*cf.* Stern, 1932, 1]. On the other hand, the mould did not always grow satisfactorily, as already mentioned, and the appearance of the animals, which justified this conclusion being drawn, certainly seemed to show that the supply of the B-factor was not at an optimum. Animals 2312 and 2315 correspond with this period of time. Unfortunately the work lacks a control group, which received the mould at its optimum growth from the start and similarly with Group 4. This latter group shows values agreeing very well with those of Group 2, and an increase compared with their corresponding animals (Group 3). The A-control animal shows a value of about the same order of magnitude. This subject will be further discussed later. Incidentally, Stern [1931] found almost equally remarkable relative rises in the K content of rabbit skins after irradiation, although the absolute values obtained were not so high. But here the irradiation had led to fairly heavy inflammation. He also found definite increases in the K values in human skin diseases (normal 157–367 mg./100 g.; inflamed, 312–635 mg./100 g.). Herrmannsdorfer and Jung [1932–33] found in normal skin, 136–244 mg./100 g. dry-weight, and 48–73 mg./100 g. fresh-weight, while one case of *Impetigo herpetiformis* (unpublished) showed a value of 150 mg./100 g. (fresh-weight). But even these values, resulting from inflammation, were not as high as ours.

Calcium content. This was normal in Groups 1, 3, 4 and 5, with a slight tendency to rise in Group 4, and in one Group 3 animal, 2317, which was investigated 26 days after beri-beri had supervened. But in Group 2, 4 out of the 5 animals which died of beri-beri showed a definite rise in the Ca values, while the last one showed a decline. The rise in calcium is similar to that in potassium, so that the ratio K:Ca (in equivalents) of this group is similar to that of Group 1, whereas it is considerably raised in Groups 3, 4 and 5, and in the case of one animal (1502) of Group 1. The highest Ca value is shown by No. 1482, but similar rises of this value are also quoted in the figures of Loewy and Cronheim [1932].

Magnesium. Our values for Group 1 are on the average higher than those of Loewy and Cronheim [1932] for rats, and are approximately the same as their values for guinea-pigs, and also as those of Brown [1926; 1927] for dogs, rabbits and human beings, if compared on a fresh-weight basis. It is however, possible that the subcutaneous adipose tissue accounts to a certain extent for this rise, for in two cases investigated by one of us [Herrmannsdorfer and Jung, 1932–33], more magnesium was found in the subcutaneous tissue than in the skin separated from it (see Table IV). This explanation is supported by the fact that 4 animals of Group 2, which had practically no fat, showed low Mg values compared with Group 1, when calculated on a dry, fat-free basis, but were of the same order of magnitude on a fresh-weight basis, and these animals yield values resembling those of Loewy and Cronheim [1932]. Groups 2, 3, 4 and 5 had no parallel rise of calcium corresponding with the rise of potassium but showed a rise of magnesium, so that the ratio Ca:Mg is lowered, while with the others it definitely rises.

Table IV.

	Sodium		Potassium		Calcium		Magnesium		Phosphorus		Chlorine	
Patient	cut.	subc.	cut.	subc.	cut.	subc.	cut.	subc.	cut.	subc.	cut.	subc.
Z.	596	633	167	204	57	49	7	20	79	72	205	268
S.	—	—	152	156	61	61	28	47	77	112	383	370

cut. = cutis, subc. = subcutaneous tissue. Calculated as mg./100 g. dry fat-free substance.

Total base (in milli-equivalents). In the case of Group 1, this lies at about the upper limit of this expression for animals treated with reduced air-pressure by Loewy and Cronheim [1932], and is therefore somewhat high. It is also high compared with the values of Brown (according to Heubner's [1931] calculations), for rabbits, dogs and human beings, and somewhat higher than in the remaining groups. This is not merely the result of the loss of weight, as is proved by the fact that an increase also occurred with two animals of Group 3 and a small increase in two animals of Group 2. The value is lowered in the case of No. 1485. A comparison of the values for individual cations with the total base yields several interesting conclusions (see Table V). First of all, the relatively high potassium content is concerned with a relative lowering of the other percentages. This is particularly significant in the case of calcium, which is present in approximately normal or somewhat lowered amounts when this method of calculation is employed and rather lags behind the figures of Loewy and Cronheim [1932] for rats and those of Brown and Luithlen for rabbits, dogs and human beings (according to Heubner's [1931] calculations). Furthermore Jung [1931, 1] and Herrmannsdorfer and Jung [1932–33] found quite different ratio values for human beings, *viz.* sodium, 60 to 77 %; potassium, 11 to 22 %; calcium, 6 to 11 %, and magnesium, 4 to 7 %. Normal values for the percentage of calcium are only seen in four beri-beri animals and the two animals Nos. 1485 and 1482.

Table V (*a*).

Group	Rat No.	Water %	Fat %	Sodium ff.dr. mg./100 g.	Sodium M.-E. 100 g. ff.dr.	Potassium ff.dr. mg./100 g.	Potassium M.-E. % ff.dr.	Calcium ff.dr. mg./100 g.	Calcium M.-E. % ff.dr.	Magnesium ff.dr. mg./100 g.	Magnesium M.-E. % ff.dr.	Chloride ff.dr. mg./100 g.	Chloride M.-E. % ff.dr.	Phosphorus ff.dr. mg./100 g.	Phosphorus M.-E. % ff.dr.	Total bases M.-E.	Total acids M.-E. %	Excess o. bases M.-E. %
1 Yeast contr.	1485	49·3	14·9	195	8·2	353	8·9	41·8	2·1	47·1	3·9	661	18·7	812	35·5	23·1	54·2	−31
	1488	56	17·5	400	17·5	590	15	35·7	1·79	49·7	4·1	114	3·2	522	23·9	38·4	27·1	+11·3
	1507	24·7	31·1	—	—	345	8·8	31·6	1·55	46·7	3·8	—	—	457	20·1	—	—	—
	1511	52·5	16·8	310	13·5	485	12·4	36·8	1·84	42·1	3·5	112	3·2	405	15·7	31·2	18·9	+12·3
	1497	47·1	27·2	396	17·2	578	14·6	34·4	1·72	38·8	3·1	303	8·6	418	18·5	36·6	27·1	+ 9·1
	1500	56·8	18·2	376	16·4	662	18·6	36·2	1·88	55·5	4·5	225	6·4	496	21·8	41·4	28·2	+13·2
	1502	52	21·7	330	14·4	872	22·2	23	1·15	51·2	4·2	372	10·6	442	19·4	41·9	30	+11·9
Averages		**51·2**	**22·1**	**363** **(94)**	**15·8**	**587** **(153)**	**15·3**	**33** **(8·6)**	**1·6**	**47** **(12·4)**	**3·9**	**225** **(58)**	**6·4**	**457** **(102)**	**20**	**37·9**	**26·3**	**+11·6**
2 Beri-beri	1482	63·2	1·4	133	5·7	718	18·2	220	10·4	387	32	369	10·8	684	29	66·1	39·8	+26·3
	1638	68·4	1·1	537	23·	1261	32	101	5·4	23·4	1·9	336	9·4	763	37	62·3	46·4	+15·9
	1721	58·5	1·8	224	9·7	1986	50	190	9·4	22·8	1·9	278	7·8	515	23·5	71·3	31·2	+40·1
	2149	65·4	6·4	317	13·7	988	25	110	5·5	41·9	3·4	444	12·6	302	13·3	47·9	25·9	+22
	2157	64·1	1·5	282	12·3	1075	26	88	4·4	36·6	3	438	12·3	333	14·5	46·1	26·8	+19·3
	2655	62·8	2·4	224	10·6	2490	63	17	0·8	85·3	7	411	11·6	811	36	81·7	47·6	+34·1
Averages		**63·9**	**2·7**	**319** **(113)**	**14**	**1556** **(536)**	**39**	**101** **(34)**	**5**	**42** **(14·7)**	**2·5**	**382** **(134)**	**10·7**	**545** **(192)**	**25**	**61·8**	**35·6**	**+25·9**
3 Mould cured	2312	52·3	23·4	362	15·8	776	19·7	26	1·3	59·3	4·9	137	3·8	307	13·5	41·7	17·3	+24·4
	2315	58·4	18·3	478	20·8	1086	27·2	36	1·8	48	4	709	20	302	13·3	53·8	33·3	+20·5
	2317	57·5	21·1	513	22·2	1219	30·8	47	2·3	118	9·6	645	18·4	433	19·4	64·9	37·8	+27·1
Averages		**56**	**21**	**451** **(101)**	**19·6**	**1027** **(229)**	**26·2**	**36** **(8)**	**1·8**	**75** **(18)**	**6**	**496** **(110)**	**14**	**347** **(77)**	**15**	**53·5**	**26·2**	**+24**
4 Mould beri-beri	2309	65·2	1·2	372	16·2	1323	34	41	2	100	8·1	407	11·4	255	11·6	60·1	23	+37·1
	2311	64	0·7	515	22·8	1393	35·2	37	1·8	67·3	5·5	500	14·1	213	9·3	65·3	23·4	+41·9
	2314	67·5	0·6	417	17·8	1190	30·2	40	2	62·5	5·2	536	15·2	211	9·3	55·2	24·5	+30·7
Averages		**65·6**	**0·8**	**435** **(141)**	**19**	**1302** **(441)**	**33**	**39** **(13)**	**2**	**73** **(26)**	**6**	**547** **(162)**	**13·6**	**226** **(76)**	**10**	**60·2**	**23·6**	**+36·6**
5 A-control	**2477**	**65·9**	**1·4**	**323** **(106)**	**14·1**	**1472** **(485)**	**37·5**	**33** **(11)**	**1·7**	**68·6** **(22)**	**5·7**	**404** **(133)**	**11·4**	**233** **(76)**	**10·3**	**59**	**21·7**	**+37·3**

fr. = fresh, dr. = dry, ff.dr. = fat-free dry. M.-E. = Milli-equivalents. The average values in brackets are fresh-weight values in mg./100 g.

Table V (*b*).

Rat No.	Group	K : Na	K : Ca	Ca : Mg	Na : Mg	Na : Cl	Na + Cl	K : P	P : Cl	Bases/Acids
					Milli-equivalents					
	1	1·06	7·84	0·43	4·11	3·13	22	0·74	3·96	1·45
	2	3·31	5·44	3·73	5·4	1·26	24·6	1·7	2·45	1·78
	3	1·32	14·4	0·32	3·6	2·12	33·6	1·7	1·41	1·91
	4	1·77	16·8	0·32	3·2	1·4	32·5	3·3	0·76	2·55
2477	5	2·65	22·3	0·29	2·5	1·23	25·5	3·6	0·9	2·7

These figures represent averages for each group.

The last two, however, show also a corresponding alkali deficit. By rearranging the table in such a way that the calcium equivalent is taken as unity (Tables VI and VII), and the other elements brought into line with it, a relative lowering of the three other cations is shown for the four beri-beri animals which had normal Ca values, while the 5th exhibits a marked rise of these ions, and the mould-fed animals and the A-control have particularly an increase of potassium. By this method of calculation, the ratio, Na:Ca:Mg shows a remarkable constancy in all the well-grown animals, even in those that subsequently underwent rapid loss of weight, while perhaps the deficient growth in a section of our animals forms the direct basis of the observed calcium increase in these cases. With these animals, the quotient K:Ca is remarkably similar to that of the controls. The drop in the magnesium value in these cases causes a considerable rise in the quotient Ca:Mg whereas this does not vary appreciably in the other cases. Only No. 2655, whose calcium was lowered in comparison to the enormously increased potassium content, had a lowered quotient. The ratio of alkali to alkali-earth is fairly constant. It is only lowered in the two animals Nos. 1485 and 1482. We are as yet far too scantily informed regarding the significance of these quotients. Table VI shows the large variations observed, which are to some extent the result of diet and of animal species, but are due in a large measure to factors not yet understood.

Table VI.

Equivalents of bases in the fat-free dry weights.

Rat No.	Sum M.-E. 100 g.	% of sum of M.-E.				Ratios for Ca = 1				Group
		Na	K	Ca	Mg	Na	K	Ca	Mg	
1485	23·1	35·6	38·5	9	16·9	4	4·3	1	1·9	1
1488	38·4	45·6	39·1	4·6	10·7	10	8·5	1	2·3	
1507	—	—	—	—	—	—	5·7	1	2·5	
1511	31·2	43·2	39·7	5·9	11·2	7·3	6·7	1	1·9	
1497	36·6	47·0	39·9	4·7	8·4	10	8·5	1	1·8	
1500	41·4	39·6	45	4·5	10·9	8·8	10	1	2·4	
1502	42	34·3	52·9	2·8	10	12	19·3	1	3·6	
Average	**37·9**	**41·9**	**43·3**	**4·5**	**10·2**	***9·3**	***7·84**	**1**	***2·2**	
1482	61·1	8·6	27·5	15·7	48·2	0·5	1·75	1	3·1	2
1638	62·3	36·9	51·4	8·7	3	4·2	5·9	1	0·35	
1721	71·3	13·6	70·6	13·2	2·6	1	5·35	1	0·2	
2149	47·9	28·6	52·6	11·6	7·2	2·5	4·56	1	0·6	
2157	46·1	26·7	57·3	9·5	6·5	2·8	5·95	1	0·7	
2655	81·7	12·9	77·3	1	8·8	13	77	1	8·8	
Average	**61·8**	**23·5**	**61·8**	**10·8**	**5·6**	**†2·1**	**†5·7**	**1**	**†0·52**	
2312	41·7	37·9	47·2	3·2	11·7	11·8	15·4	1	3·6	3
2315	53·8	38·7	50·6	3·4	7·3	11·4	15	1	2·1	
2317	64·9	34·2	47·5	3·6	14·7	9·5	13·2	1	4·1	
Average	**53·5**	**36·9**	**49·1**	**3·4**	**11·2**	**11·7**	**14·4**	**1**	**3·3**	
2309	60·1	27	56·1	3·4	13·5	8	16·5	1	4	4
2311	65·3	34·9	53·9	2·8	8·4	12·5	19·1	1	3	
2314	55·2	32·3	54·8	3·6	9·3	9	15	1	2·6	
Average	**60·2**	**31·4**	**54·9**	**3·3**	**8·8**	**9·5**	**16·6**	**1**	**3·2**	
2477	59	23·9	63·6	2·8	9·7	8·5	22·3	1	3·5	5

* Without 1502. † Without 2655.

Table VII.

Author	Animal species	Treatment	Ratios for Ca = 1 (Milli-equivalents)			
			Na	K	Ca	Mg
Fawns-Jung	Rat	Yeast-control	4·0–12·0	4·3 –19·3	1	1·8–3·6
		Beri-beri	0·5–13	1·75–77	1	0·2–0·8
		Mould-fed	9·5–11·8	13·2 –15·4	1	2·1–4·1
		Mould-fed beri-beri	8 –12·5	15 –16·5	1	2·6–4
		A-avitaminosis	8·5	22·3	1	3·5
Loewy-Cronheim [1932]	Rat	Normal	4·2– 5·8	1·8–4·8	1	0·7–1
		Diluted air	8 –16·2	1·6–2·9	1	0·6–1·4
		Irradiated	6	1·9	1	1·1
		Dark	3·9– 5·1	3·7–4·6	1	1·4–1·7
Börnstein [1926]	Mouse	—	—	1·6–1·92	1	—
Hayashi [1926]	Rabbit	Normal	7·5	4·7	1	0·8
		Cabbage-fed	7	4·4	1	0·9
		Oats-fed	7·5	6·5	1	0·9
		HCl-fed	5·6	6	1	0·9
		$CaCl_2$ injection	5·6	3·5	1	0·6
Kaplansky [1928]	Rabbit	Normal	1 –2·6	0·5–1·6	1	—
		Irradiated	0·7–4	0·8–2·7	1	—
Stern [1931]	Rabbit	Normal	—	2 –5·4	1	—
		Irradiated	—	2·5–5·9	1	—
Brown [Heubner, 1931]	Rabbit	Normal averages	2·5	1·23	1	0·95
	Dog	Normal averages	4·2	2·84	1	1·06
	Man	Ill averages	6·8	2·65	1	1·06
Herrmannsdorfer-Jung [1932–33]	Man	Tuberculosis	6·8–12·3	1·2–2·7	1	0·2–0·78
Jung-Marx. Not published	Woman	*Impetigo herpetiformis*	—	3·5	1	0·4
Nathan and Stern [1928]	Man	Normal averages	—	5·4	1	—
		Dermatosis averages	—	6·1	1	—

Chlorine content. The chlorine content of Group 1 is very low. This might be attributed to the Cl content of the subcutaneous connective tissue [*cf.* Loewy and Cronheim, 1932]. This however cannot be generalised as the following facts seem to indicate that subcutaneous tissue need not necessarily have a much lower chlorine content than skin itself. Firstly, in Group 3, two animals which had a high fat content had higher chlorine values, while the other three had less; secondly, no noteworthy difference in chlorine content between cutis and subcutis was found in the two human skins cited in Table IV. Jung [1931, 1] was able to evolve a ratio for contrasting the plasma and connective tissue in musculature, by which it was established that in some cases the connective tissue contained an equal amount of chlorine to the plasma, in others less, but in a few cases significantly more. In the well-known work of Wahlgren [1909] and Padtberg [1910], the former found for dogs 376 mg./100 g. Cl in skin *plus* fatty tissue, and the latter 269 mg./100 g. in skin less fat. Atchley and Benedict [1927] give 132–240 mg./100 mg. (fresh-weight) as normal for dogs. Stern [1931] has recently shown that the skin can assimilate chlorine, for after administering "Afenil" ($CaCl_2$, urea) to rabbits, a marked rise of the skin Cl content was observed, whereas its calcium did not increase. The skin did not alter appreciably in its appearance, and there was no corresponding increase in water content. In one case in which the Cl content rose from 380 mg. to 1080 mg./100 g., the water, in fact, dropped from 73·3 % to 70·1 %. So also with our animals, no parallel relation is shown between the water and chlorine contents, but it does occur between water and sodium chloride contents (the sum of Na and Cl equivalents), the fat contents being identical. In relation to the water content, fat appears to be more important than salt (NaCl), and further, chlorine can be retained "dry," but not sodium chloride.

It is noteworthy that the highest figures for the sodium chloride equivalent occur in the two groups of mould-fed animals, which as already mentioned showed an enormous thirst and great sensitivity to lack of water. But a large water requirement is generally observed with all animals participating in vitamin-B tests, especially in summer. Lack of water often produces bleeding, especially *via* the urogenital tract, which disappears immediately on ensuring an adequate supply of water. This sensitivity is seldom observed among animals being used for other investigations. The quotient Na:Cl varies somewhat irregularly.

Phosphate content. Only the non-ether-soluble phosphorus was estimated. But under this heading come many substances in which phosphorus is held in organic combination. Now since both the nucleic acids and the nucleotides as well as the hexosephosphoric acids have a dissociable hydrogen atom in the phosphoric acid group, they must be taken into account as regards acid-base regulation, whereas this is hardly the case with the phosphatides. One should therefore be justified in placing the various non-ether-soluble phosphoric fractions together and calculating them as acid. The calculation as equivalents is worked out at neutral reaction according to the system of Reichel and Spiro [1927]. The phosphorus generally shows a tendency to sink in the case of the beri-beri animals, especially in Group 4, but in Group 2 only two animals show a significant drop, while with two others, on the contrary, there is a rise. This is perhaps a result of poor growth since the phosphate value is also distinctly increased in the deficiently grown animals, 1482 and 1485.

The ratio P:Cl exhibits wide variations, if anything with a tendency to drop in Groups 2, 4 and 5, against which the ratio K:P shows a rise of about double the amount between Groups 1 and 2, and 3 and 4 respectively, while 3 has similar values to 2 and 5 to 4. The unusual animals, 1485 and 1482, have much diminished quotients.

Total acid content. The total acid shows no particular variations. A rise is seen in a few animals of Groups 2 and 3. It is particularly high in animal 1485.

Excess base. An increase in the amount of excess base is shown by Group 2 relative to Group 1, and by Group 4 relative to Group 3. The A-control has a value of the same order of magnitude as Group 4.

One single animal, No. 1485, shows a considerable excess of acid.

DISCUSSION.

In comparison with the control animals, the most striking change occurs in the potassium content, which is considerably increased in the beri-beri groups. This is not merely the result of emaciation, since the animals of Group 3 had equally high values, even in the presence of a good layer of fat, while one animal of Group 2 had a relatively low value. The values are higher than any yet obtained, even from skins affected by inflammation, and the relative increase corresponds to those obtained by Stern [1932, 2] from inflamed human skin and from the skin of irradiated rabbits as compared with normal skin. But with those of our animals taken for analysis, a visible change of the hide only occurred with No. 2655 which had a damp, rough skin shortly before the onset of beri-beri, and with the animals of Group 4, the hides of which were always rough. No such signs of inflammation were observable at the time of the investigation. It should be mentioned that the high potassium content is significant as a preparatory step in the skin prior to an inflammatory reaction, for in the case of the mould-fed animals remaining in the experiment after the others had been removed for analysis, a deterioration of the skin was apparent, and about a month later quite a severe dermatitis occurred. It is noteworthy that the A-control animal, 2477, reacted in almost the same way as the Group 4 animals and to some extent like those of Group 2. Although only one single animal is conçerned, the high potassium content is so striking that a discussion of the possible reasons for this is justified. Firstly, it should be mentioned that out of 14 animals receiving the same diet as No. 2477, in the vitamin A experiments, 9 developed a dermatitis 11–25 days after the commencement of the feeding and one of them indeed, only one day after. It is interesting that, sooner or later, a spontaneous healing took place, and the hair grew again. This was observed also in the case of three animals receiving 2 mg. cod-liver oil or $2 \cdot 5 \gamma$ carotene in olive oil per head. This tends to refute the theory that the dermatitis is due to a vitamin B deficiency in the basal diet and is cured when the supplementing substances are administered, for, as previously emphasised, the symptoms first appeared after the supplementary feeding was commenced.

The symptoms somewhat resemble those found by Burr and Burr [1929; 1930], which, together with the results of Burr and Brown [1931], were attributed to a deficiency of highly unsaturated fatty acids of high molecular weight, a suggestion which was disputed by Gregory and Drummond [1932]. In this deficiency disease, however, the dermatitis occurs more on the tail and hind-quarters, but with our animals almost exclusively on the foreparts, and only in one case in a vitamin B_1 investigation on the haunches, though this never occurred at all in the vitamin A experiments.

In its localisation it somewhat resembled the "pellagra" appearance. But it lacks the localisation around the eyes, previously observed [Jung, 1931, 2], and the clinical picture did not correspond with the description by Chick and Roscoe [1927; 1928], Findlay [1928], Sherman and Sandels [1929], and Scheunert and Lindner [1927]. Moreover, this dermatitis occurred in one animal that increased to 185 g. in 52 days, while receiving 0·1 g. yeast daily in a vitamin B_1 test. With one mould-fed animal, the dermatitis was cured by administration of egg-white, but in another case this proved ineffective.

It is therefore conjectured that as a result of a deficiency of any essential factor of diet, the skin becomes sensitive, and a dermatitis occurs as a result of subsequent metabolic changes (with the supplementary feeding). This is supported by the fact that spontaneous healing takes place concurrently with a satisfactory weight increase. It is not claimed that the changes of the skin observed with the various vitamin B groups and the vitamin A control are due to the same cause, for Nathan and Stern [1928] found identical disturbances in dermatoses of the most varying kinds, and Portmann [1927] found identical changes in the skin histologically after vitamin A-free, vitamin B-free and quantitatively insufficient diets.

One should at least comment on the extremely similar observation here, to that of Burr and Burr [1930], namely, the great thirst of our mould-fed animals and their sensitivity to lack of water. The skin of these animals showed to a certain extent an increase in the sodium, chlorine and water contents, which exhibited a relationship between the water content and the sum of Na + Cl, but independent of either of them taken singly. This relationship is in any case only valid under identical conditions of fat-content.

Have these changes anything to do with the deficiency of vitamins B_1 and B_4? It seems possible to answer this question in the positive and to state with justification that in rat beri-beri, the potassium content of the skin rises considerably, with small changes in the absolute values for sodium and magnesium, whereas to a certain extent the calcium alters correspondingly in such a way that the ratio K:Ca tends to remain constant. It is noteworthy that the specific animals which showed a markedly rough hide exhibited no rise in calcium. Phosphorus was definitely lower in Group 4 than in Group 3, but in the beri-beri Group 2, this was only the case with a few animals and then in relation to calcium. There is therefore a rise in the ratio K:P in most of these animals. It is unfortunate that it was not possible to estimate phosphorus in the ether extract as well, as one cannot tell if any change occurred in the lipin phosphorus, for, among others, Schmitz and Hiraoka [1928] have observed relationships between beri-beri and phosphatide metabolism.

The variations in the chemical constituents of the skin dealt with in this paper do not seem to be specific for beri-beri but are rather an expression of general changes in metabolism.

Whether or not this is due to changes in the internal secretions must at present be left an open question.

SUMMARY.

1. The mould, *Aspergillus niger*, can form vitamin B_1 from a vitamin B-free diet and cure and prevent beri-beri in rats. The vitamin is mostly contained in the conidia (spores) and only to a small extent in the mycelium and the basal diet on which the mould is grown.
2. Animals nourished on this mould-covered food appear nervously excitable and have a large water requirement and great sensitivity to lack of water.
3. The mineral content of the skin, after ether-extraction, was determined in groups of growing rats on various diets with the object of ascertaining the changes due to beri-beri.
4. In beri-beri a marked increase in potassium was found, and in some cases a corresponding rise of calcium. This is not the result of deficient growth, for it was much less marked in one rat that had no beri-beri.
5. Magnesium is raised wherever no corresponding calcium increase follows a rise in potassium, so that the ratio of univalent to bivalent bases is fairly constant.
6. A relationship has been shown between the sum of the equivalents of sodium and chloride and the water content, under similar conditions of fat content.
7. Phosphate generally showed a tendency to sink in beri-beri, chloride to rise.
8. A vitamin A control animal showed approximately the same ratio values as the beri-beri animals. In this connection it should be mentioned that a dermatitis was often observed in vitamin A tests, occurring first after supplemental feeding. Spontaneous healing occurred as weight increased, the condition thus resembling the beri-beri dermatitis.
9. Possible explanations for this are discussed.

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CXXI. SOME CONSIDERATIONS REGARDING AND INVESTIGATIONS INTO CALCIUM AND PHOSPHORUS METABOLISM.

By JOHN POOL McGOWAN.

(*Aberdeen.*)

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During an investigation into the nature of vitamin D action, the results of which are recorded elsewhere [McGowan *et al.*, 1931; McGowan, 1933], certain phenomena were observed with regard to Ca and P metabolism which were deemed worthy of further inquiry; a perusal of the literature supplied others, and an attempt has been made in this investigation to answer some of the questions arising in this way.

It is desirable, for clarity, first to discuss briefly certain factors which play important parts in the matter under investigation.

Gastric acidity. If one assumes that calcium is absorbed either as organic calcium salts occurring in the food or as calcium chloride formed by the interaction of calcium salts, such as the carbonate of the food, with the gastric hydrochloric acid, whilst phosphoric acid is absorbed in the form of its salts with alkali metals or of acid salts of the alkaline earths, it is evident that the gastric acidity must exercise an important influence over the absorption of Ca and P.

So long as the amount of the gastric hydrochloric acid normally excreted is in excess of the total uncombined base and carbonates of the food, conditions will be favourable for solubility, and therefore complete absorption, of Ca and P. If on the other hand, the gastric juice is insufficient to neutralise all the calcium (and alkali) carbonates of the food, whilst a moderate proportion of the calcium may be converted into calcium chloride and absorbed, the phosphates will be precipitated as tricalcium phosphate to an extent depending on the excess of calcium carbonate and to this extent will escape absorption.

It appears, therefore, that, contrary to Stewart and Percival [1928] and others, a deficient absorption of Ca can occur only in response to absolute deficiency of Ca in the diet (so far at least as dietary composition alone is concerned) whilst excess of Ca in the diet can prevent absorption of an otherwise adequate amount of P. Optimum conditions for absorption and subsequent utilisation of Ca and P will be obtained when these are supplied in the diet in moderate and approximately equivalent amounts, with the proviso that the total uncombined base of the food must be considerably less than sufficient to neutralise the gastric hydrochloric acid[1]. As regards utilisation, if Ca or P is rapidly absorbed in large quantity into the blood, utilisation will be affected in that, the body being unable to "fix" such large amounts, these will be immediately excreted in order to keep the composition of the blood normal.

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[1] The bearing of the condition "achlorhydria" on the present subject will be discussed in a subsequent communication.

Intestinal excretion of Ca and P. In spite of the lack of quantitative data on normal animals, it has been firmly established [*cf.* Mendel and Benedict, 1909–10; Walsh and Ivy, 1927–28; Stewart and Percival, 1928] that Ca and P are actually excreted by the intestine: in dogs the amount of Ca excreted in this way is said to be independent of that taken in the diet [Taylor and Weld, 1932], whilst in herbivora the intestinal excretion of tricalcium phosphate forms an important mechanism for regulating acid-base equilibrium.

Ca and P in the blood. It is not clear that calcium phosphate really exists in supersaturated solution in the blood as has been claimed [Holt, La Mer and Chown, 1925; Peters and Van Slyke, 1931]: the failure of calcium phosphate to precipitate from blood-serum in spite of the fact observed by Adler [1919], that precipitation of calcium phosphate occurs from an artificial "inorganic" blood-serum, is probably rather to be explained by the effect of the serum-proteins in diminishing ionisation [Shear and Offner, 1931; *cf.* also Pauli and Schon, 1924]. The action seems to be one of preventing the formation of the insoluble calcium phosphate rather than of keeping it in solution after it has been formed. Whatever the nature of the mechanism may be, however, which normally prevents precipitation of calcium phosphate from blood-serum, owing to the solubility properties of this salt, it is evident that any influence, such as increase in Ca or P concentration which would tend to bring about its precipitation, will be more effective at an alkaline reaction.

In point of fact under ordinary conditions the normal alkaline state of the blood is usually associated with access of considerable quantities of calcium to the blood and hence with the usually found high Ca : low P relationship (the reverse ratio is produced experimentally when blood-Ca is lowered by injection of alkaline phosphates [Binger, 1917]). On the other hand, in conditions of acidity of the blood such as is produced by the administration of calcium chloride, there is an increase both in the Ca and P concentrations [Salvesen *et al.*, 1924; Collip, 1926, 1, 2], a state of affairs which also occurs in the terminal stages of hyperparathyroidism and which may be brought about artificially by the simultaneous injection of $CaCl_2$ and acid sodium phosphate [Collip, 1926, 1]. Thus in acid states of the blood there is no fixed ratio between the Ca and P concentrations.

The claim of Howland and Kramer [1921] that the maintenance of the Ca × P product of the blood above a certain level is to be regarded as diagnostic of freedom from rickets may be considered in this connection. The ascription of any significance to this product[1] is open to theoretical objection since no account is taken of the state of combination and active concentrations of the elements under consideration: moreover, the results of other workers [Greenwald, 1931; Farquharson and Tibbets, 1931; Stearns and Knowlton, 1931; Dupré and Semeonoff, 1931; Bourne and Campbell, 1932; Theiler and Green, 1932; present investigation] show that the Ca × P product cannot in fact be regarded as in any sense an index of normality, as has been suggested by Howland and Kramer [1923]. No attempt has been made to explain the frequent abnormal increases in the Ca × P product which may be observed, and the view that the product defines the physiological state of the organism in any way becomes manifestly absurd when we consider that identical values may be found for it in normal and abnormal animals with the ordinary high Ca and low P ratio and in the tetanic animal with low Ca and high P.

Such "reciprocal" relationship as may exist between the Ca and P of the blood appears rather to be an expression of a balanced action between calcium and phosphorus compounds in the blood, the point of equilibrium of this action being determined by the effect of the reaction of the blood on the solubility of the least soluble of these compounds (calcium phosphate) which it contains.

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[1] Considerable confusion has crept into this subject by forgetting, in reading blood-Ca and -P concentration charts, that Ca × P involves a geometric not an arithmetic conception.

In all work involving determinations of Ca and P in blood it is necessary to remember that the concentrations of these elements actually observed are the resultants of a large number of different processes such as absorption, excretion, endogenous formation and fixation, and without consideration of these factors, particularly that of diet [*cf.* Bourne and Campbell, 1932; Dupré and Semeonoff, 1931] the figures may have little or no significance.

EXPERIMENTAL OBSERVATIONS.

A series of CaO and P_2O_5 balance experiments was carried out to test the effect of the amounts of CaO and P_2O_5 and their ratio in the food on their retention and excretion.

Full-grown rabbits, weighing 2000 to 2300 g. were employed, the experiments being carried out in the usual way with the usual precautions. The stock ration consisted of equal parts of bran, sharps and "Porage" oats, to 24 lbs. of which was added 1 lb. of fish meal. For roughage, 100 g. turnip were given each rabbit daily and no account was taken of this in the balance. Water was freely available.

The composition of the various rations fed are given in Table I.

Table I.

No.	Description	CaO %	P_2O_5 %	Ratio CaO/P_2O_5
1	Stock ration with high $CaCO_3$ content	4·5589	1·6193	2·8
2	,, "balanced" with $CaCO_3$	1·1512	1·8476	0·62
3	,, + steamed bone flour ($Ca_3(PO_4)_2$)	4·5325	4·7772	0·95
4	,, + dicalcium phosphate	3·0829	5·0303	0·60
5	Stock ration	0·4200	1·7883	0·23
6	,, *minus* fish meal	0·0970	1·4848	0·065
7	,, + medium Na_2HPO_4	0·0432	3·2204	0·013
8	,, + high Na_2HPO_4	0·0130	4·4695	0·0029

It will be seen that the ratio CaO/P_2O_5 diminishes considerably from ration 1 to ration 8 in the table. As the animals exercised some degree of selection from the ration, the ratios given here for the food offered differ slightly from those of the food actually eaten (Table II).

Except where otherwise stated, the rabbits were fed on the experimental ration for a week before collection of urine and faeces for analytical purposes was commenced. The results of the various experiments will be discussed together and are collected in synoptic form for this purpose, in Table II, in six groups: the figures are average daily ones.

Column A represents the CaO/P_2O_5 ratio in the food eaten in the various experiments. This differs slightly, owing to selection, from that in Table I but diminishes in the same way from group I through group VI to group V.

Column O shows that the amount of P_2O_5 consumed in the various experiments is high and, with the exception of the steamed bone flour and dicalcium phosphate experiments, roughly equal. The diminishing CaO/P_2O_5 ratio series is due therefore to diminishing calcium intake as is shown in column G.

The CaO balance follows the same diminishing order except in the case of rabbit *C*, on steamed bone flour, where a high negative balance is in all

Table II.

			P_2O_5						CaO								
Rabbit	Dates	Days	Intake nett g.	Urine g.	%	Faeces g.	%	Balance g.	Intake g.	Urine g.	%	Faeces g.	%	Balance g.	Food eaten ratio CaO to P_2O_5	Fraction intake P_2O_5 excreted in urine	Group
	Q	P	O	N	M	K	I	H	G	F	E	D	C	B	A	X	
Stock ration + high $CaCO_3$:																	
A	8–12. vii. 31	5	0·8089	0·0472	7·15	0·6128	92·85	+0·1480	1·6159	0·7976	63·2	0·4645	36·8	+0·3738	1·99	0·05	I
B	7–12. vii. 31	6	0·9684	0·0088	1·3	0·6584	98·7	+0·3012	2·2889	0·4040	35·8	0·7254	64·2	+1·1595	2·3	0·009	
C	25–30. vii. 31	6	0·9354	0·0123	1·4	0·8831	98·6	+0·0400	2·1233	0·6572	45·1	0·7989	54·9	+0·6672	2·2	0·01	
C^1	13–26. x. 31	14	0·8535	0·0398	4·5	0·8347	95·5	−0·0210	1·9434	0·5421	44·5	0·6752	55·5	+0·7261	2·2	0·04	
H	29. xi.–11. xii. 31	13	1·0883	0·0346	2·8	1·1810	97·2	−0·1273	2·3557	0·5679	36·1	1·0069	63·9	+0·7809	2·08	0·03	
H^1	18–21. xii. 31	4	1·2898	0·0567	5·7	0·9414	94·3	+0·2917	2·8036	0·9338	52·8	0·8348	47·2	+1·0350	2·17	0·04	
Stock ration "balanced" with $CaCO_3$:																	
E	28. x.–9. xi. 31	13	1·3622	0·2359	18·9	1·0092	81·1	+0·1171	0·7034	0·0274	4·8	0·5458	95·2	+0·1301	0·49	0·17	II
Stock ration + steamed bone flour:																	
C	2–5. viii. 31	4	0·8657	0·2282	20·8	0·8668	79·2	−0·2293	0·3214	0·1479	18·8	0·6396	81·2	−0·4661	0·37	0·26	VI
G	2–11. xii. 31	10	1·9882	0·2857	13·4	1·8450	86·6	−0·1424	1·3718	0·1376	10·8	1·1390	89·2	+0·0952	0·69	0·14	
Stock ration + dicalcium phosphate:																	
G	12–23. xii. 31	12	1·8500	0·4909	22·9	1·6566	77·1	−0·2975	0·9416	0·0415	4·3	0·9128	95·7	−0·0127	0·50	0·26	
Stock ration:																	
C	17–22. vii. 31	6	1·2075	0·4881	49·9	0·4902	50·1	+0·2292	0·2148	0·0390	16·2	0·2033	83·8	−0·0275	0·17	0·40	III
C^1	30. ix.–12. x. 31	13	0·8368	0·3767	47·5	0·4160	52·5	+0·0441	0·1660	0·0115	6·6	0·1620	93·4	−0·0075	0·19	0·45	
D	30. ix.–12. x. 31	13	1·2849	0·2212	24·9	0·6656	75·1	+0·3981	0·2280	0·0041	1·2	0·3512	98·8	−0·1273	0·17	0·18	
G	17–27. xi. 31	11	1·3051	0·4264	40·1	0·6370	59·9	+0·2417	0·1478	0·0110	5·1	0·2039	94·9	−0·0671	0·11	0·32	
Stock ration less fish meal:																	
D	13–21. x. 31	9	1·0856	0·2626	22·1	0·9240	77·9	−0·1010	0·0694	0·0018	0·6	0·2942	99·4	−0·2266	0·07	0·24	IV
G	10–21. i. 32	12	0·9825	0·2893	33·6	0·5699	66·4	+0·1233	0·0391	0·0026	1·4	0·1848	98·6	−0·1483	0·04	0·29	
H	10–21. i. 32	12	1·3681	0·4989	38·0	0·8125	62·0	+0·0567	0·0564	0·0024	1·1	0·2234	98·9	−0·1694	0·04	0·36	
Stock ration + medium Na_2HPO_4:																	
G	22. i.–2. ii. 32	12	0·7973	0·2718	38·1	0·4422	61·9	+0·0833	0·0298	0·0024	1·8	0·1299	98·2	−0·1025	0·037	0·33	
Stock ration + high Na_2HPO_4:																	
G	3–9. ii. 32	7	0·8682	0·2338	52·7	0·2097	47·3	+0·4247	0·0015	0·0014	1·8	0·0766	98·2	−0·0765	0·0018	0·27	V

probability due to the animal having been on a high $CaCO_3$ diet immediately previously (Ca fixed lightly as $CaCO_3$).

The percentage excretion of CaO in the urine—column E—has the same trend; the percentage excretion of CaO in the faeces—column C—is the inverse of this. The slight irregularity in the lower figures of these series is probably due to difficulty of accurate estimation of the very small quantities of CaO involved.

Particular attention is directed to the very high positive CaO balance in the high $CaCO_3$ experiments, which is coupled with a low P_2O_5 balance and a high percentage excretion of P_2O_5 in the faeces. This indicates two things, first, that CaO can be fixed in large quantities in the body without the aid of P_2O_5, and second, that a large quantity of P_2O_5 is rendered insoluble in the intestine by $CaCO_3$ and hence is not absorbed. The alternative to this latter conclusion, namely that the insoluble $Ca_3(PO_4)_2$ is absorbed and then re-excreted into the intestine, hardly merits discussion.

In dealing with the P_2O_5 metabolism, the experiments may be divided into six groups according to the CaO/P_2O_5 ratio and the P_2O_5 balance, as follows: group I, high $CaCO_3$ ration, P_2O_5 balance definitely positive, but low; group II, "balanced" $CaCO_3$ ration, P_2O_5 balance definitely positive; group III, stock ration, P_2O_5 balance definitely positive; group IV, stock ration *minus* fish meal, and *plus* medium Na_2HPO_4, P_2O_5 balance definitely positive; group V, stock ration *plus* high Na_2HPO_4, P_2O_5 balance definitely positive; and group VI, steamed bone flour and dicalcium phosphate ration, P_2O_5 balance very negative.

There are thus five groups, I to V, showing a positive P_2O_5 balance; and an outside group, group VI (placed where it is in Table II because of the CaO/P_2O_5 content of the ration), in which calcium phosphate is being fed, with a very definite negative P_2O_5 balance.

The results obtained in groups I and II show that, in spite of a fairly high $CaCO_3$ content of the ration, rendering P_2O_5 insoluble and unabsorbable, enough P_2O_5 has been taken up, as alkaline phosphates, to give rise to a positive balance: group V, on the other hand, demonstrates that a large positive balance of P_2O_5 may occur even when the food contains little CaO and indicates that P_2O_5 may be fixed in the body, as is easily understandable, largely in other forms than in association with CaO. The results of groups III and IV point, though in less degree, in the same direction.

The findings in group VI are of great interest. In considering them, the figures for rabbit *C* are not taken into account, for, although the results are of the same order, this rabbit, immediately preceding the experiment was on a high $CaCO_3$ ration, a circumstance reflected in the high negative CaO balance. The results in the two rabbits left in this group may be compared with those obtained in the rabbit in group II. The rations in the two groups have approximately the same CaO/P_2O_5 ratio, but the rabbits in group VI are consuming considerably more P_2O_5 than that of group II. Nevertheless, the P_2O_5 balances in group VI are markedly negative, while that in group II is positive. This is associated with the fact that the Ca and P in the ration of group VI are in the form of calcium phosphate while, in group II, they exist as $CaCO_3$ and food phosphorus, independent of one another. Results, somewhat similar in nature, were obtained by Tereg and Arnold in dogs [1883].

The same state of affairs is reflected in column X of Table II, where the amount of P_2O_5 passed in the urine is expressed as a fraction of the intake in the food. In group VI the negative balance has occurred in spite of the large intake of P_2O_5 and although a very considerable fraction of this intake has actually passed through the body of the animal and been excreted in the urine.

It seems therefore that, if a positive balance is to be regarded as evidence of utilisation, regard being had to the P_2O_5 and CaO balances in these groups, calcium attached to phosphorus in the form of calcium phosphate is of much less value to the animal economy than it is in circumstances where the Ca and P are supplied uncombined with one another, in the form of salts such as $CaCO_3$, $CaCl_2$ *etc.*, and Na_2HPO_4 *etc.* Because of the volatility of the H_2CO_3 of $CaCO_3$ on neutralisation and the consequent loss of acidity of the intestinal contents, salts of calcium with non-volatile acids would also appear to be preferable.

The moderate and approximately equal positive balances of both CaO and P_2O_5, obtained in group II, support the view, expressed in the introduction (*vide supra*), regarding the optimum conditions for ensuring the not too rapid simultaneous absorption of CaO and P_2O_5, namely as to their being present in moderate and approximately equivalent amounts.

Attention may be directed to the very large positive CaO balances in group I in connection with the subject of normal Ca and P metabolic activities of herbivora. The conditions present here, with the excretion of large quantities of calcium as $CaCO_3$ and small amount of phosphorus in the urine *etc.*, duplicate these to a considerable extent and need not be further described.

A few remarks may be made on the bearing of the results obtained in the present investigation on the subject of rickets. The characteristic finding in the blood in this disease is a considerable decrease in P in the serum [Iversen and Lenstrup, 1919; Howland and Kramer, 1921; Hess, 1930]. Shohl, Brown, Rose, Smith and Cozard [1931] have shown that an alkalotic condition of the blood occurs in rickets. This, together with an absolute deficiency of P in the diet or one produced by excess of calcium in the diet[1], would have a tendency to keep at a low level the P in the blood available for metabolism; while such curative and preventive measures as fasting and the administration of vitamin D [Kramer, Shear and Siegel, 1931; McGowan *et al.*, 1931], by supplying phosphate as well as acid endogenously, would, by keeping P in solution in quantity in the blood, have an opposite effect.

In the introductory discussion, allusion was made to the theory that $Ca_3(PO_4)_2$ circulates in a state of supersaturation in the blood, and the difficulties in accepting such a hypothesis were remarked on. It remains to state briefly how some of the difficulties especially in regard to calcification and decalcification may be overcome by the theory advanced in this communication. The vital activity of the osteoblasts would be capable of producing, locally where required, H_2CO_3 and H_3PO_4 for the precipitation of Ca from the $CaCl_2$, circulating in the blood under the protection of its proteins: while the osteoclasts, by reversing the process and breaking up $CaCO_3$ and $Ca_3(PO_4)_2$ of the bones, would supply $CaCl_2$ and NaH_2PO_4 to the blood, to be protected as before from interaction and kept in solution by the blood-colloids.

During the course of the investigation the Ca and P concentrations in the blood were estimated in certain cases. The taking of the samples could not be regulated with regard to the time of feeding as the animals consumed their ration at all times. As, however, the animals were bled at the same time each day—between 11 and 12 a.m.—and, as the routine of the experiments otherwise was identical, the results are roughly comparable. Table III supplies them, arranged in relation to the CaO/P_2O_5 ratio of the food eaten.

Table III shows that, while there may be a tendency for some of the animals on a diet with high CaO/P_2O_5 ratio to show a high Ca concentration in the blood and for the average to be higher than that of those on a low CaO/P_2O_5 ratio diet,

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[1] This point will be discussed in a subsequent communication.

Table III.

No.	Rabbit	Date	Ca mg./ 100 cc.	P mg./ 100 cc.	CaO/ P_2O_5	Ca × P	Group	Diet
1	*C*	16. x. 31	16·7	2·81	2·2	46·92	I	High $CaCO_3$
2	*D*	25. ix. 31	13·1	3·96	—	51·87		”
3	*H*	4. xii. 31	19·0	3·55	—	67·45		”
4	*H*	10. xii. 31	16·3	3·39	—	55·25		”
5	*E*	5. xi. 31	13·7	4·0	0·49	54·8	II	“Balanced” $CaCO_3$
6	*G*	4. xii. 31	14·5	3·75	0·50	54·37	VI	Tricalcium phosphate
7	*G*	10. xii. 31	18·2	3·55	—	64·61		”
8	*G*	17. xii. 31	14·1	3·97	—	55·97		Dicalcium phosphate
9	*C*	7. x. 31	15·6	3·38	0·17	52·72	III	Stock
10	*D*	21. ix. 31	12·4	3·97	—	49·22		”
11	*D*	23. ix. 31	12·5	3·93	—	49·12		”
12	*D*	7. x. 31	13·4	4·07	—	54·53		”
13	*G*	24. xi. 31	12·7	4·14	—	52·57		”
14	*N*	2. xi. 31	15·1	3·07	—	46·35		”
15	*D*	16. x. 31	13·1	4·22	0·04	55·28	IV	Stock less fish meal
16	*G*	18. i. 32	12·2	3·73	—	45·5		”
17	*H*	18. i. 32	14·6	3·54	—	51·68		”
18	*G*	26. i. 32	14·2	3·54	0·037	50·26		Medium Na_2HPO_4
19	*G*	8. ii. 32	12·3	4·16	0·0018	51·16	V	High Na_2HPO_4

the difference is not striking. There is no significant or consistent difference in the P concentration of the blood in the different groups. There is no evidence of the existence of a reciprocal relationship between the Ca and P concentrations, and the Ca × P product, varying as it does between 45·5 and 67·45, shows little sign of constancy, that is in absolutely healthy animals under identical conditions and with but slight differences in diet and indeed even when on the same diet.

SUMMARY AND CONCLUSIONS.

The results obtained, in so far as they concern calcium in the ration, have special reference to conditions where calcium is supplied as $CaCO_3$. There seems to be no reason, however, why they should not be applicable, if in less degree, to situations where calcium is supplied in other forms.

The most important single factor controlling the absorption of Ca and P appears to be the acidity of the gastric juice: subject to this condition, the most important factor influencing the absorption of P seems to be an excess of Ca in the diet, which, having neutralised the HCl, precipitates P as $Ca_3(PO_4)_2$.

The optimum conditions for the simultaneous absorption of Ca and P seem to be those where, in the presence of adequate HCl, CaO and P_2O_5 exist in the diet in moderate and approximately equivalent amounts and in such quantity that the amount of Ca is considerably short of that required for the neutralisation of the HCl.

For the optimum utilisation of Ca and P after absorption, it seems to be requisite that they should be supplied in the food uncombined with one another and that they should be absorbed as far as possible in this condition.

The Ca and P salts of the blood seem to be prevented from interacting with one another and to be held in solution by the proteins; an "acid" condition of the blood, by increasing the solubility, will assist in this. High concentration of P in the blood is usually, in natural conditions, associated with an "acid" state; and here analysis will show Ca and P concentrations in various proportions.

Where, however, the reaction of the blood has an "alkaline" tendency, as occurs in normal and alkalotic conditions, chemical activity, more or less restrained previously, asserts itself and a balanced reaction occurs, with a point of equilibrium towards the side of the element—Ca or P—the salts of which are present in the greater concentration; and, as under such conditions $Ca_3(PO_4)_2$ is precipitated and calcium salts usually predominate, analysis will show a high concentration of Ca relatively to that of P.

Under such circumstances, there seems to be no warrant for the assumption that, in general, there exists a reciprocal or inverse relationship between the Ca and P concentrations of the blood.

Howland and Kramer's index of normality appears also not to be valid because, apart from the considerations just mentioned, the Ca × P product is not in fact a constant in normal individuals or even in healthy animals kept under fairly normal and identical conditions and fed on the same diet or on slight variations from this.

It is pointed out that the concentrations of P and Ca in the blood are the resultants of the factors of absorption, excretion and endogenous formation and fixation. If this is taken into consideration, along with the effect of the balanced reaction in "alkaline" conditions as just described, it will be seen how difficult it is to correlate Ca and P concentrations of the blood with other co-existent phenomena. The fact that the concentrations of Ca and P bear no relationship to the chemical activities of the elements as they are combined in the blood also requires emphasis in this connection.

Although the rapid absorption of quantities of Ca or P is followed by their rapid excretion in order that the composition of the blood may be kept within normal limits, considerable amounts of Ca may be fixed in the body in conditions where there is little P absorbed and *vice versa*.

The possible relation of the results obtained to a theory of the causation of rickets, namely deficiency of P, is discussed.

I wish to express my indebtedness for considerable help rendered by Mr William Godden. Along with his assistants Mr W. H. Williams and Miss B. W. Simpson, he carried out the various analyses on which this investigation is based. Dr D. W. Auchinachie and Mr A. R. G. Emslie supplied the blood analysis figures.

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CXXII. FURTHER INVESTIGATIONS INTO THE FUNDAMENTAL NATURE OF VITAMIN D ACTION.

By JOHN POOL McGOWAN.

(*Aberdeen.*)

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In a preliminary paper [McGowan, Cunningham and Auchinachie, 1931] certain tentative conclusions were drawn, on the basis of incomplete balance experiments, regarding the intrinsic and essential nature of vitamin D action. A series of complete calcium and phosphorus balance experiments has since been carried out, and these are dealt with in this paper.

These experiments were conducted in the customary way on rabbits in metabolism cages, the usual precautions being taken with regard to feeding and collection of urine and faeces. As the animals, in eating their ration, selected certain parts of it, care was taken to recover all the non-eaten portion, and the calcium and phosphorus intake was determined from its analysis. Water was freely available. For roughage, 100 g. turnip was given daily. This was not taken account of in the eventual balance. As a general rule, the faeces and urine were collected daily and were analysed, as far as possible, in 2-day periods.

The basis of the ration fed was 8 parts each of "Porage" oats, white sharps and bran, together with 1 part of fish meal. In some cases, the fish meal was withdrawn, while, in others, $CaCO_3$ was added. The composition of the various rations fed as regards CaO and P_2O_5 was as follows:

Table I.

	CaO %	P_2O_5 %
1. Rabbit *A*	4·5589	1·6193
2. ,, *B*	4·5589	1·6193
3. ,, *C* (stock)	0·4200	1·7883
4. ,, *C* ($CaCO_3$)	4·5589	1·6193
5. ,, *D* (stock)	0·4200	1·7883
6. ,, *D* (*minus* fish meal)	0·0970	1·4848
7. ,, *E* ($CaCO_3$)	1·1512	1·8476
8. ,, *H* ($CaCO_3$)	4·5589	1·6193
9. ,, H^1 (*minus* fish meal)	0·0970	1·4848

Rabbit A, buck, weight 2130 g.; ration fed consisted of stock ration as above, to 25 lbs. of which were added 2 lbs. $CaCO_3$ (Table I, 1). The experiment lasted from 8 to 12. vii. 31 (pre-period). It received 4 cc. of a solution of irradiated ergosterol (1,000,000 rat units per cc.), on 12. vii. 31, and the same dose on 13 and 14. vii. 31. It was killed on 15. vii. 31. The results of the balance are given in Table II.

The points to be noted in Table II are the very marked negative P_2O_5 and CaO balances and the shift of the excretion of P_2O_5 from the faeces to the urine in the experimental period. In the pre-period, the urine, as voided, was very alkaline and contained a large deposit of $CaCO_3$ crystals: while in the experimental period the urine was acid and clear, becoming turbid on cooling owing to separation of phosphates.

Table II*.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
A			P_2O_5				
Pre-period	8–12. vii. 31 (5 days)	0·8090	0·0472	7·15	0·6128	92·85	+0·1480
Exp. period	13–15. vii. 31 (3 days)	0·1212	0·3223	30·2	0·7440	69·8	−0·9451
			CaO				
Pre-period	8–12. vii. 31 (5 days)	1·6159	0·7976	63·2	0·4645	36·8	+0·3738
Exp. period	13–15. vii. 31 (3 days)	0·261	0·4155	51·0	0·3993	49·0	−0·5538

* All figures are average daily figures.

Rabbit B, buck, weight 2365 g., fed on same ration as rabbit A (Table I, 2); pre-period lasted from 7 to 12. vii. 31; experimental period from 13 to 16. vii. 31. On 12. vii. 31, it received, subcutaneously, at 10.45 a.m., 2 cc. $CHCl_3$. The results of the balance are given in Table III.

Table III.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
B			P_2O_5				
Pre-period	7–12. vii. 31 (6 days)	0·9684	0·0088	1·3	0·6584	98·7	+0·3012
Exp. period	13–16. vii. 31 (4 days)	0·2653	0·1425	12·8	0·9743	87·2	−0·8495
			CaO				
Pre-period	7–12. vii. 31 (6 days)	2·2889	0·4040	35·8	0·7254	64·2	+1·1595
Exp. period	13–16. vii. 31 (4 days)	0·6387	1·0781	55·3	0·8726	44·7	−1·3121

The same points in regard to the balance, shift of P_2O_5 from faeces to urine and urinary deposit, as noted in regard to rabbit A, are in evidence here. There is also a shift of the CaO excretion from the faeces to the urine, not observed in rabbit A.

Rabbit C, buck, weight 2855 g. This rabbit has two pre-periods—the first, from 30. ix. 31 to 12. x. 31, being on the stock ration (Table I, 3); and the second, from 13 to 26. x. 31, on the stock ration *plus* $CaCO_3$ (Table I, 4). The experimental period lasted from 27 to 30. x. 31. 50,000 rat units of irradiated ergosterol were given on 26. x. 31; 100,000 on 27 and 28. x. 31; 50,000 on 29. x. 31; and 50,000 on 30. x. 31, when the rabbit was killed. The results of the balance are given in Table IV.

In comparing the second pre-period and the experimental period, the same points, in general, are to be noted as in the two previous cases. There is here also a shift of CaO excretion from the faeces to the urine, a condition present in rabbit B but not in rabbit A.

Table IV.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
C			P_2O_5				
Stock ration	30. ix. 31–12. x. 31 (13 days)	0·8368	0·3767	47·5	0·4160	52·5	+0·0441
Stock ration $+CaCO_3$	13–26. x. 31 (14 days)	0·8535	0·0398	4·5	0·8347	95·5	−0·0210
Exp. period	27–30. x. 31 (4 days)	0·3734	0·2935	22·5	1·0104	77·5	−0·9305
			CaO				
Stock ration	30. ix. 31–12. x. 31 (13 days)	0·1660	0·0115	6·6	0·1620	93·4	−0·0075
Stock ration $+CaCO_3$	13–26. x. 31 (14 days)	1·9434	0·5421	44·5	0·6752	55·5	+0·7261
Exp. period	27–30. x. 31 (4 days)	0·7984	1·7227	69·2	0·7646	30·8	−1·6889

When the animal was killed, the p_H of the blood was found to be somewhat lowered at 7·3; its alkali reserve was lower also, corresponding to 1·68 cc. $N/100$ NaOH per 1 cc. (normal = 2·8 to 3 cc.); blood-P was 6·75 mg./100 cc.; Ca, 14·3 mg./100 cc.

Rabbit D, buck, weight 1900 g. There were two pre-periods here also, the first, from 30. ix. 31 to 12. x. 31, on the stock ration (Table I, 5), the second, from 13 to 21. x. 31, on this stock ration, *minus* fish meal (Table I, 6). The experimental period, when vitamin D was given, was on the latter ration and lasted from 22 to 26. x. 31. On 21. x. 31, it received 1,000,000 rat units of irradiated ergosterol and the same dose on 22. x. 31 and 23. x. 31; on 24. x. 31, 500,000 rat units and none on 25. x. 31. The results of the balance are given in Table V.

Table V.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
D			P_2O_5				
Stock ration	30. ix. 31–12. x. 31 (13 days)	1·2849	0·2212	24·9	0·6656	75·1	+0·3981
Stock ration less fish meal	13–21. x. 31 (9 days)	1·0856	0·2626	22·1	0·9240	77·9	−0·1010
Exp. period	22–26. x. 31 (5 days)	0·1910	0·5158	62·0	0·3157	38·0	−0·6405
			CaO				
Stock ration	30. ix. 31–12. x. 31 (13 days)	0·2280	0·0041	1·2	0·3512	98·8	−0·1273
Stock ration less fish meal	13–21. x. 31 (9 days)	0·0694	0·0018	0·6	0·2942	99·4	−0·2266
Exp. period	22–26. x. 31 (5 days)	0·0125	0·0323	23·6	0·1043	76·4	−0·1241

Here again there is a marked negative balance and a shift of the excretion of the P_2O_5 and CaO from the faeces to the urine. The urine, which was clear and acid in the pre-periods, became very turbid with phosphates in the experimental period. The p_H of the blood, when the animal was killed on 26. x. 31, was 7·3, the alkali reserve, expressed as previously, gave a figure of 2·58 cc. $N/100$ NaOH per cc.; the blood-P was 12·99 mg./100 cc. and Ca 11·7 mg./100 cc.

Rabbit E, buck, weight 2310 g. After it had been kept on the ordinary stock ration for some time, the pre-period, which lasted from 28. x. 31 to 9. xi. 31, was conducted on a ration in which the P_2O_5 was roughly balanced in regard to the CaO by the addition of $CaCO_3$ (Table I, 7) (by adding 4·8 oz. $CaCO_3$ to 25 lbs. of the stock ration). The experimental period lasted from 10 to 16. xi. 31. 50,000 rat units of irradiated ergosterol were given on 9, 10, 11, 12, 13, 14, 15 and 16. xi. 31. The results of the balance are given in Table VI.

Table VI.

Rabbit and period	Extreme dates	Intake nett g.	Output: Urine g.	Output: Urine %	Output: Faeces g.	Output: Faeces %	Balance g.
E			P_2O_5				
Pre-period	28. x. 31–9. xi. 31 (13 days)	1·3622	0·2359	18·9	1·0092	81·1	+0·1171
Exp. period	10–16. xi. 31 (7 days)	0·8524	0·5146	55·2	0·4184	44·8	−0·0806
			CaO				
Pre-period	28. x. 31–9. xi. 31 (13 days)	0·7033	0·0274	4·8	0·5458	95·2	+0·1301
Exp. period	10–16. xi. 31 (7 days)	0·4678	0·1548	41·5	0·2181	58·5	+0·0949

In this case there is a lowering of the CaO and P_2O_5 balances and a shifting of the excretion of the CaO and P_2O_5 from the faeces to the urine. The effects here, however, are much less than in the more or less comparable (as regards dosage of vitamin D) rabbit C, for which the proportion of $CaCO_3$ in the diet is much greater, an occurrence which appears to be the only outstanding difference between the two cases in experimental conditions. The urine, during the experimental period, was very turbid with phosphates.

When the animal was killed on 16. xi. 31, the p_H of the blood was 7·3; alkali reserve = 1·35 cc. $N/100$ NaOH per 1 cc.; blood-P = 7·5 mg./100 cc. and Ca = 15·95 mg./100 cc.

Rabbit H, buck, weight 2320 g. As one of the major effects of the administration of large doses of vitamin D to the rabbit is to produce a loss of appetite and a diminution in the intake of food, it became necessary to try to elucidate the effect of starvation alone on the excretion of P_2O_5 in the rabbit. This was carried out in the present experiment. The rabbit was fed on the calcium-rich diet (Table I, 8); and, as complete starvation had never occurred in any of the rabbits poisoned with vitamin D, a certain very limited amount of food, comparable with the amount taken in these latter cases, was allowed. The pre-period lasted from 29. xi. 31 to 11. xii. 31, and the experimental period from 12. xii. 31 to 17. xii. 31. The results are given in Table VII.

The main point to notice in this case is not so much the balances or the shift of excretion of P_2O_5 and CaO from the faeces to the urine but the very small

Table VII.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
H			P_2O_5				
Pre-period	29. xi. 31– 11. xii. 31 (13 days)	1·0883	0·0346	2·8	1·1810	97·2	−0·1273
Starvation	12–17. xii. 31 (6 days)	0·2933	0·0938	14·8	0·5381	85·2	−0·3386
			CaO				
Pre-period	29. xi. 31– 11. xii. 31 (13 days)	2·3557	0·5679	36·1	1·0069	63·9	+0·7809
Starvation	12–17. xii. 31 (6 days)	0·5933	0·3664	48·3	0·3925	51·7	−0·1656

absolute amount of P_2O_5 that is excreted in the urine daily during the starvation period. This should be compared with the large absolute amount present in the urine in the vitamin D cases. These occurrences are indicative of an endogenous source for the large excess present in the latter cases. It should be noted also that a large amount of the calcium, absorbed in the pre-period, is excreted in the urine in the starvation period, as carbonate, however, and not as phosphate as in the vitamin D cases.

Rabbit H^1, buck, weight 2100 g. This experiment was undertaken to test the effect of irradiation, as an alternative to irradiated ergosterol administration, on the Ca and P metabolism. Rabbit H was again used, after a considerable rest. The ration consisted of the stock ration, less fish meal (Table I, 9). The hair on the back, over an area of about 12 square inches, was kept closely shaved and the skin, thus exposed, irradiated daily for a period of 20 minutes at a distance of 2½ feet from a powerful "Hanovia" mercury vapour lamp. The pre-period lasted from 10 to 21. i. 32 and the irradiation period from 22. i. 32 to 9. ii. 32. The results are given in Table VIII.

Table VIII.

Rabbit and period	Extreme dates	Intake nett g.	Output				Balance g.
			Urine		Faeces		
			g.	%	g.	%	
H^1			P_2O_5				
Pre-period	10–21. i. 32 (12 days)	1·3681	0·4989	38·0	0·8125	62·0	+0·0567
Irradiation period	22. i. 32– 9. ii. 32 (19 days)	0·9309	0·4850	52·6	0·4364	47·4	+0·0095
			CaO				
Pre-period	10–21. i. 32 (12 days)	0·0564	0·0024	1·1	0·2234	98·9	−0·1694
Irradiation period	22. i. 32– 9. ii. 32 (19 days)	0·0392	0·0023	2·2	0·1033	97·8	−0·0664

The results obtained here are not so striking quantitatively as in the majority of the vitamin D poisoning cases. This is natural as the treatment has been much less drastic. Leaving out of consideration the calcium results on account of the smallness of the amount in the diet and in the excreta, especially the urine, the results are of the same order as those obtained in vitamin D poisoning. There is less food eaten in the experimental period than in the pre-period; but, in spite of this, there is the same amount of P_2O_5 excreted in the urine as in the pre-period, there is a shift of excretion of P_2O_5 from the faeces to the urine and the P_2O_5 balance is diminished.

The experiments have been carried out according to the accustomed routine, and, in the tables as submitted, the data obtained are arranged in the traditional fashion to show the state of the balance, the shift of the phosphorus from the faeces to the urine *etc.* The results, however, must be interpreted with care, as the designation of some of the experiments as "balance" experiments would appear to be a misnomer.

In many of the experiments, from the very outset after the administration of the vitamin D, the taking of food by the animal practically ceased; the gastro-intestinal functions were completely upset; the food, consumed on the last day of the pre-period, remained undigested and unabsorbed in the intestine; there was no evacuation of faeces[1]: while the large amount of $CaCO_3$, present in the diet in some cases, would lead to difficulties by neutralising the HCl of the gastric juice, and it would also tend to precipitate and retain in the intestine the phosphates derived from the food. These occurrences would all tend to obviate a real balance being struck; while, from the practical aspect, it needs to be mentioned that the "faeces," in most of the cases in the experimental period, had to be obtained from the dead animal by scraping out the contents of the intestines, those of the stomach, however, not being included.

Any attempt, therefore, to draw conclusions, under the circumstances, from the state of the "balance" as given in the Tables would (in most of the cases) be out of place. At the same time attention should be directed to the high degree of negativity, as regards CaO and P_2O_5, that did occur in these cases, a circumstance to which significance is added by the fact that those rabbits (*D*, *E*, and H^1), which were subjected to less drastic treatment (less CaO in food or less vitamin D administered), and which consequently lived for a longer period and were more normal in their excretory activities, exhibited a similar, if less marked, negativity in their CaO and P_2O_5 balances.

The shift of the P_2O_5 excretion from the faeces to the urine, which occurred in every case, has a very definite significance as will be seen from the following considerations. One recognises the fallacy underlying any attempt, under the circumstances of the stagnation of the intestinal contents *etc.*, to place reliance on this ground alone on the percentage figures of "shift" as given in the Tables; but if one compares the absolute amounts of P_2O_5 in the urine of the animals in the experimental periods with that in their urine in the pre-period and with that in the urine of a healthy rabbit in a similar grade of starvation, one is on much firmer ground, and the figures for the "shift" become significant. In the first case, when the pre-period and experimental period are compared, in spite of the fact that practically no food is being taken in the experimental period, the absolute amount of P_2O_5 in the urine is greatly, sometimes enormously, increased; while, in the second case of equal degree of starvation (though not necessarily of equal degree of want of absorption from the intestines in the case of the "healthy" rabbit), the amount of P_2O_5 excreted in the urine of the "healthy" starved rabbit is only a small fraction of that excreted by those poisoned with vitamin D. Only one conclusion would seem to be valid under the circumstances, namely that the excess P_2O_5 in the animals poisoned with vitamin D must be of endogenous origin, and it has already been suggested [McGowan *et al.*, 1931] that its source is the lipins of the body which are broken down under the influence of the vitamin D. It should be noted also that the amount of P_2O_5 actually passing by the urine does not give an exact criterion of the extent of this splitting up, for a considerable amount of the phosphorus, so formed, is fixed in the tissues of the body in an insoluble form in the various "calcifications."

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[1] The difficulties in regard to possible excretion of Ca and P into the intestine are appreciated. In the present instance, these are of no moment as subsequent discussion will show.

The results obtained in the rabbit poisoned with $CHCl_3$ (rabbit *B*) and in the one treated by irradiation (rabbit H^1) are the same in type as those just discussed and require no special discussion.

The peculiar circumstances attending the performance of such experiments in regard to the starvation during the experimental period, as has just been discussed, make it possible to derive the same conclusions, as have been arrived at in the present instance, also from the results obtained in the preliminary investigation [McGowan *et al.*, 1931], where urinary samples alone were analysed.

The behaviour of calcium in the present investigation, being of less importance, is not discussed at length. It plays a secondary rôle to the phosphorus, being drawn from the intestines or endogenous sources to neutralise the phosphoric acid set free by the action of vitamin D. Where $CaCO_3$ has been fed in large amounts in the pre-period, the large positive balance of loosely bound calcium forms an easily available endogenous source. Attention may also be drawn to the fact that, while rabbits on a $CaCO_3$-rich diet excrete a large part of the Ca as $CaCO_3$ in the urine, when poisoned with vitamin D the calcium appears in the urine as calcium phosphate. As the animals during this latter period are in a condition of starvation, this may be accepted as further evidence of vitamin D producing the phosphoric acid from an endogenous source and of calcium acting as an anti-acid as already indicated.

In the course of the investigation some observations were made on the calcium and phosphorus contents of the blood. The results are given in Table IX.

Table IX.

Rabbit	Date	Ration	Experimental treatment	Ca mg./100 cc.	P mg./100 cc.
C	7. x. 31	Stock	None	15·6	3·38
	16. x. 31	Stock + $CaCO_3$	None	16·7	2·81
	29. x. 31	Stock + $CaCO_3$	Vitamin D	15·3	6·01
	30. x. 31	Stock + $CaCO_3$	Vitamin D	14·3	6·75
D	7. x. 31	Stock	None	13·4	4·07
	16. x. 31	Stock *minus* fish meal	None	13·1	4·22
	26. x. 31	Stock *minus* fish meal	Vitamin D	11·7	12·99
E	5. xi. 31	Stock + $CaCO_3$	None	13·7	4·0
	13. xi. 31	Stock + $CaCO_3$	Vitamin D	13·0	5·72
	16. xi. 31	Stock + $CaCO_3$	Vitamin D	15·95	7·5
H^1	18. i. 32	Stock *minus* fish meal	None	14·6	3·54
	22. i. 32	Stock *minus* fish meal	Irradiation	15·9	4·19
	26. i. 32	Stock *minus* fish meal	Irradiation	15·9	4·21
	1. ii. 32	Stock *minus* fish meal	Irradiation	15·7	4·25
	8. ii. 32	Stock *minus* fish meal	Irradiation	15·9	4·63

The only significant feature in this table is a definite and consistent rise of the blood-P, following the administration of vitamin D. Because this rise has taken place in spite of the lack of absorption and of the greatly increased excretion of P by the urine the result has significance and supports the main thesis. Too much emphasis however should not be laid on such increases, for they constitute the resultant of several processes, absorption and excretion, together with endogenous fixation and formation; and it is quite possible, to give an illustration, that in the present instance, with a different relative participation of the different factors, a lowering of P concentration in the blood might have been obtained, even although there was no abatement of endogenous formation.

The figures for P and Ca concentration in the blood indicate an activity somewhere, but, being of the nature of a resultant, give no information as to its essential *locus* or nature, unless the factors producing this resultant are known and to each is apportioned its due meed. The figures in Table IX show no consistent rise in the calcium content of the blood although others, *e.g.* Ashford [1930], Warkany [1930], Naser [1930], *etc.*, have found a rise in the blood-calcium following the administration of vitamin D. Such findings, however, are not necessarily contradictory; for vitamin D may be producing its essential action without, for the reasons mentioned above, the index appearing in the blood, a view opposed to the narrow one fairly commonly held, that vitamin D affects the blood directly and has as chief function the regulation of its Ca and P content, as an end in itself.

SUMMARY.

The results obtained in this extended investigation confirm those found in the preliminary inquiry and go to show that vitamin D produces its action by setting free inorganic phosphate endogenously and in all probability from the lipins of the body, for which evidence was supplied previously.

I wish to record my great obligation to Mr William Godden in connection with this investigation. He, with his assistants Mr W. H. Williams and Miss B. W. Simpson, carried out the analysis of the foods, faeces and urine in the balance experiments. My thanks are also due to Dr D. W. Auchinachie and Mr A. R. G. Emslie for the blood analyses. I am indebted to Mr A. L. Bacharach, Glaxo Laboratories, for supplying the solution of vitamin D.

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CXXIII. OBSERVATIONS ON SOME FACTORS IN THE MILK OF INDIVIDUAL COWS WHICH MODIFY THE GROWTH OF LACTIC STREPTOCOCCI.

By HUGH ROBINSON WHITEHEAD and GEOFFREY ALAN COX.

From the Dairy Research Institute, Massey Agricultural College, Palmerston North, New Zealand.

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It is well established that there are relatively wide variations in the chemical composition of milk taken from individual cows. Differences in the biological properties of individual milks have also been reported from time to time although these have never been correlated with chemical differences with certainty. From the point of view of those interested in the manufacture of cheese and butter, the most important way in which biological variations in milk are manifested is in their effect upon the growth and metabolic activity of the lactic acid bacteria and particularly upon the growth of the lactic streptococci which are commonly added to milk in an attempt to direct along certain lines the biological changes which take place in the manufactured products. Most of the papers that have been published on this question deal with the effect of particular samples of milk on the flavour of "starter" cultures (mixed cultures of lactic streptococci) produced when such milk was used as the bacteriological medium. Baker and Hammer [1926] found that the milk from certain cows produced "starter" cultures which were abnormal in flavour and aroma. They also noticed differences in the final acidity developed in the various milk samples by a given culture. They were unable to find any correlation between acid development and flavour, but they noticed that, in general, milk with a high content of total solids tended to permit the development of high final acidity. Knudsen and Sørensen [1929] also observed that milk containing a high percentage of total solids permitted the development of a high titratable acidity by lactic streptococci in 24 hours and suggested that the higher concentration of buffer substances in such milk explained the more active growth of the bacteria. They also found that certain samples of milk which had been drawn aseptically and sterilised immediately would not readily support the growth of certain strains of lactic streptococci until yeast extract or hydrolysed milk had been added. Gorini [1929] made a similar observation. Kelly [1930] noticed a very definite and constant difference in the flavour of a "starter" culture when it was propagated in the milk of two different herds; he took no account however of acid development. Farmer and Hammer [1931] found that starter cultures varied in their resistance to the germicidal action of raw milk and that different milks varied in their action on different cultures. The effect of the germicidal action which was still evident in milk kept at 68° for 30 minutes was no longer present when the milk was kept at 71° for 30 minutes. With regard to germicidal or restraining power there seems to have been doubt for some considerable time as to whether the action was due to a germicidal substance or whether the various cells in milk were responsible. The work of Jones [1928] seems to show beyond doubt that a restraining substance quite distinct from blood complement exists in milk and incidentally that the substance varies in concentration from time to time in the milk of individual cows and even in milk from the different quarters of a single cow.

It is evident from these scattered observations that the growth of lactic streptococci in a medium composed partly or wholly of milk may be restrained through several causes.

(i) In raw milk or in milk heated only to a low temperature the germicidal action may lead to a delay in the growth of the organisms.

(ii) In some samples of milk there may be lacking certain substances which are necessary for the rapid growth of the bacteria. Even a low percentage of total solids may apparently lead to a check in acid development at some stage owing to insufficient buffering.

(iii) The cells which vary in number in the milk both of healthy and of diseased cows may restrain or destroy the bacteria by phagocytic action.

The influence of bacteria, present in milk as a contamination, on the subsequent growth of streptococci is not considered here, for it is intended to deal only with those factors which are present in the milk as it leaves the cow's udder.

The work to be described was originally undertaken with the object of determining whether any of these influences were of significance in the acid production which takes place in rennet curd during the process of cheese manufacture. There is sometimes great difficulty in promoting a sufficiently rapid rate of acid development in the curd in a cheese vat; and although this is often undoubtedly due to a lack of vitality in the lactic streptococci themselves, it seemed possible that the properties of the particular batch of milk used to form the curd might on occasions play a part. The results to be described show that this is indeed the case.

The test which was applied to different batches of milk consisted in the addition of a standard amount of a 24-hour "starter" culture, a treatment of the milk in somewhat the same manner as in the process of cheese manufacture, and a determination at definite times of the acidity of the whey which exuded from the curd. This procedure was followed in preference to the simpler method of determination of the acid production in the milk itself, because it was found that greater differences occurred in the acidities of different wheys than in the batches of whole milk, thus rendering the detection of variations more easy. In general the streptococci give lower acidities in a given time in whey from curd than in the corresponding whole milk. The reason for this will be discussed later. It also seems true that any inhibitory factor makes its influence more strongly felt in curd and thus leads to the greater differences which are observed. Moreover, the method used here demonstrates mainly the effect of restraining (or accelerating) factors in the milk on the rate of acid development in the initial and middle stages of the growth of the streptococci, in distinction to the methods of other workers, which without exception deal with the total acid development over a period of 18 to 24 hours. In the process of cheese manufacture it is the rate of acid development in the curd which is most important; hence the advisability of using a test which simulates the actual conditions and which enables one to determine the rate of acid development rather than the total capacity for acid development.

METHOD.

150 cc. of milk in a beaker were adjusted in temperature to 30°, and 1·5 cc. of a 24-hour culture of lactic streptococci in milk were added. The beaker was kept at 30° for 30 minutes, after which time 0·2 cc. of a rennet solution was added. The beaker was maintained at 30° for 1 hour to allow coagulation to take place, and then the curd was cut with a knife as evenly as possible into pieces about ½-inch square. The mixture of curd and whey was heated to 37°, and after 2 hours at that temperature the whey was drained from the curd as completely as possible. The beaker containing the curd was replaced in the 30° incubator for 2 hours. The concentration of acid in the whey which had exuded was then determined by titration, and all the whey was again drained off. After a further hour at 37° the whey which had exuded from the curd was again titrated. The acidities expressed as parts per thousand of lactic acid were taken as measures of the absolute quantities of acid produced and the difference between the two readings as the rate of acid development. It was found by experiment that differences of the order of 0·5 might occur between the results of duplicate tests, and that comparisons between tests on several batches of milk were justified only when all the tests were performed on the same day, mainly because of variations in the vitality of the "starter" culture from day to day.

RESULTS.

The individual raw milks of several cows were tested by this method. The milk was drawn from the cows direct into sterile vessels, an approximately equal quantity being taken from each quarter of the udder. The samples were placed immediately in a refrigerator and were used in the test within 2 hours of having been drawn. Microscopic examination showed that on every occasion the milks contained less than 10,000 organisms per cc. (the lower limit detectable by direct microscopic count). Since the cultures of lactic streptococci subsequently added contained on the average one billion cocci per cc. it was assumed that the number of contaminant acid-forming organisms present in the samples was in no case sufficient to influence the results of the acidity test. The following results were obtained in the first test (Table I). It will be observed that most of the milk samples gave substantially the same result, but that the milk from Cow T was outstanding in that the curd prepared from it gave practically no acid development whatever. On further test it was found that several cows in the herd gave milk which showed a similar inhibition of acid development. Upon investigation it was found that all the cows giving this abnormal milk had at some time suffered from mastitis, although at the time the test was made they were considered healthy. In some instances a considerable time had elapsed between recovery from the disease and the date of this test.

Table I.

	Acidity as parts per 1000 lactic acid	
Name of cow	1st reading	2nd reading
OD	2·7	5·7
R	2·5	4·5
G	2·2	4·2
WD	2·5	5·4
A.F.	3·2	6·6
K	2·4	5·1
I	2·6	5·7
T	1·1	1·2
L	2·7	6·3
F	2·6	5·7

Microscopic examination of the milk used in the acidity tests showed however that whenever the phenomenon of inhibition occurred large numbers of leucocytes were present. Three cows which gave such abnormal milk were selected for testing purposes, and samples of milk from the individual quarters of their udders were taken on several occasions. The samples were subjected to the acidity test and to a reductase test (by the addition of methylene blue and incubation at 37°) and the numbers of cells present were estimated by the method of Breed [1911]. Table II gives typical results.

Table II.

Cow	Quarter	Cells per cc.	Reductase time (hrs.)	Acidities as parts per 1000 lactic acid	
				1st reading	2nd reading
L.C. Pure-bred Friesian	R. hind	640,000	>7	2·1	4·5
	L. hind	>60,000,000	$\frac{3}{4}$	1·2	1·4
	R. fore	370,000	>7	2·0	4·2
	L. fore	530,000	>7	2·3	4·2
T. Grade Jersey	R. hind	2,410,000	>7	1·9	3·9
	L. hind	>10,000,000	2	0·8	0·9
	R. fore	180,000	>7	2·3	3·8
	L. fore	1,970,000	>7	1·8	3·9
G. Grade Jersey	R. hind	5,550,000	$4\frac{1}{2}$	1·4	2·3
	L. hind	140,000	>7	2·3	3·6
	R. fore	110,000	>7	2·5	3·9
	L. fore	70,000	>7	2·3	3·6

Many results such as these make it evident that there was an accurate correlation between a cell count above a certain value, a reduction of methylene blue within 7 hours and an inhibition of acid development in the rennet curd. The phenomenon was usually limited to milk from a single quarter of each abnormal cow, but on continued trial it was found that the condition was not constantly associated with any one quarter. Thus in the case of Cow T the following results were obtained on consecutive days:

Date	Abnormal quarter
7. viii. 32	L. hind
8. viii. 32	None abnormal
9. viii. 32	R. fore
10. viii. 32	R. hind
11. viii. 32	L. hind

On a subsequent day the milk from the L. fore quarter was also abnormal. The results of many tests on several cows showed that a cell count of five million per cc. was approximately the lower limit at which the phenomenon appeared. On odd occasions a milk containing fewer cells (of the order of two or three millions per cc.) gave a curd which inhibited the development of acid, but the inhibition was constantly found only when the numbers exceeded five millions. The milk of Cow T was examined daily for several months, and during that period it showed the phenomenon of inhibition at irregular intervals although at no time did the animal suffer from an acute attack of mastitis. The milk was normal also in appearance throughout the whole period, although the person who milked the cow occasionally observed small lumps during the process of milking. The cause of the inhibition became evident when a culture of lactic streptococci was added to a sample of milk which contained abnormal numbers of cells and the mixture incubated at 37° for a few hours. A stained smear of the milk examined under the microscope showed almost complete phagocytosis of the streptococc with consequent inhibition of their growth or fermentive activity. In some samples the organisms ultimately overcame the retarding effect of the cells and showed renewed growth. This was also evidenced in the acidity tests when the curd samples were allowed to remain in the incubator overnight. Some of the curds which had shown practically no acid development within the normal period of the test then gave a considerable development of acidity. Others which contained much larger numbers of cells still gave very little acid development. No attempt was made to differentiate the various types of cells in the milk samples, but certainly over 90 % of the cells in samples containing several millions per cc. were polymorphonuclear leucocytes. It seems to be quite definite from the results described that the leucocytes were sometimes able by their phagocytic action to prevent the development of streptococci for periods longer than 24 hours, while at other times they were capable of delaying activity for at least 6 hours.

Effect of heat upon abnormal milk.

It was of interest to determine what heat treatment of milk containing large numbers of cells sufficed to remove its inhibitory action on lactic streptococci. Milk which is pasteurised before consumption by human beings is usually heated at 63° for 30 minutes, while milk which is to be used for the manufacture of cheese is, in New Zealand, usually heated to 66–70° for 30 seconds. When abnormal milk such as has been described was heated for 30 minutes at 63° the inhibitory effect was entirely removed and normal results were obtained in the acidity test. Incubation of the heated milk with an addition of lactic streptococci showed also that phagocytic action was entirely absent.

Some experiments were then performed in which milk containing abnormally large numbers of cells was kept at various temperatures for a much shorter time, approximately 30 seconds, as is the practice in pasteurising milk by the flash process. The milk was run through the condensing space of a double surface condenser through the outer and inner jackets of which steam was passed. The rate of flow of the milk was controlled so that the desired temperature was recorded on a thermometer with its bulb immersed in the stream issuing from the lower end of the condenser. A second condenser through the outer jacket of which cold water flowed served to cool the milk immediately to a temperature of 30°. It was thus shown by means of acidity tests on abnormal raw milk and on the same milk pasteurised that the limiting temperature at which the inhibitory action was removed was with most samples 49–52°. After treatment at this temperature the majority of the samples tested still showed a slight inhibitory action, but at any higher temperature the milk after treatment gave a result in the acidity test which was within the normal range. Table III gives a set of typical results.

Table III.

		Acidity as parts per 1000 lactic acid	
Treatment		1st reading	2nd reading
Milk containing 24×10^6 cells per cc.	Raw	0·9	0·9
	Pasteurised 63° 30 mins.	2·5	5·4
	Pasteurised 49–52° 30 secs.	1·4	3·0
Normal milk	Pasteurised 63° 30 mins.	2·3	4·2

It is evident therefore that any of the heat treatments of milk normally used in practice would eliminate the inhibitory effect of leucocytes on the development of acidity in rennet curd. Even in raw milk it is unlikely that the average number of cells in a mixed supply would be great enough to exert a marked effect on the growth of lactic streptococci, and as will be shown in a later section of this paper a number of cells less than that which gives phagocytosis is likely to lead to a stimulating rather than an inhibiting effect.

Differences between the milks of apparently normal cows.

During the course of the work on the milk of abnormal cows it was noticed that there were some cows whose milk, although normal in respect of its content of cells, yet gave consistently low results in the acidity test. The check in acid production was by no means so marked as with milk containing abnormal numbers of cells, but it was remarkably steady with any one animal over a period of many days. In the absence of any possibility of marked phagocytic action the possible explanations seemed limited to a deficiency in the milk or curd considered as a medium for the streptococci, or to an inhibitory factor in the milk. Six normal cows were selected as representative of those giving high and low results and samples of their milk were subjected daily to the acidity test. It was realised that the "starter" culture to be used would probably vary in activity from time to time, so two control milks were also used, one consisting of the mixed milk from the whole herd of about 50 cows and the other of a milk prepared from a dried skim milk. For the latter control one batch of powder was used throughout the whole period of the experiment. Each milk was tested both in the raw state and after pasteurisation at 63° for 30 minutes. Table IV gives particulars of the cows, and the graphs in Fig. 1 show the results obtained.

Table IV.

Cow	Breed	Average fat in milk %	Days between date of calving and beginning of experiment
1	Grade Jersey	4·8	77
2	Grade Jersey	5·1	54
3	Pure Friesian	3·4	227
4	Pure Friesian	3·2	76
5	Grade Friesian	3·2	111
6	Grade Jersey	4·5	60

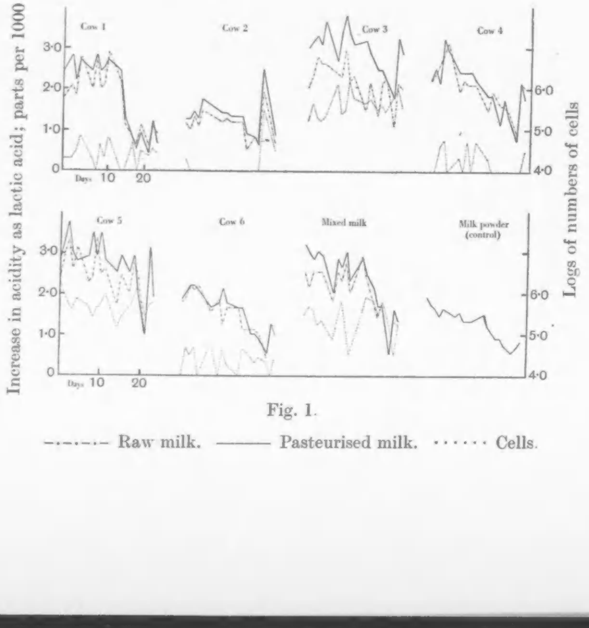

Fig. 1.

–·–·–·– Raw milk. ——— Pasteurised milk. ······ Cells.

For simplicity only the increases in acidity between the first and second readings in the test are plotted, so that the results really indicate the rate of acid production in the curds at an arbitrarily chosen time after the start of the test. The numbers of cells in all the milk samples were estimated by the direct microscopic count as used for the counting of bacteria in milk and the logarithms of the number of cells are also plotted on the graphs. A consideration of the graphs leads to several interesting conclusions.

(*a*) The milk from any single cow tended to maintain a certain general characteristic level in rate of acid production in the curd in spite of slight fluctuation from day to day.

(*b*) With the milks of most of the cows pasteurisation tended to lead to a slightly higher rate of acid production. This is in agreement with the observations of Benson and Evans [1913]. It is probably due in part to a destruction of the bactericidal substance present in raw milk.

(*c*) The milks from individual Jersey cows gave curds in which the rate of acid development was in general lower than the rate in curds from Friesian milk. At first sight this seems opposed to the findings of Baker and Hammer [1926] who stated that milk with a high total solids content (*e.g.* Jersey milk) developed a higher acidity in 24 hours than milk with a low total solids content. There is however no real discrepancy, for it is found that although the acid in the moisture in Jersey milk curd develops at a slower rate during the cheese making process it ultimately reaches a higher value than the acid in the moisture of Friesian milk curd if sufficient time is allowed. The different rates of acid development in Jersey and Friesian curds are probably due to differences in moisture content. The greater the percentage of fluid in the medium, the more rapid will be the growth of the bacteria, and it is well known that curd prepared from milk of high solid content is less rich in moisture and even loses that moisture more readily than curd from milk of lower total solid content, all other conditions being equal. The same factor (*i.e.* difference in moisture content) probably explains the more rapid production of acid by bacteria in whole milk than in any type of curd.

(*d*) The curves showing the numbers of cells present in the milk samples show a tendency to follow the curves showing the rates of acid development. The tendency is evidenced both in general level and in many cases also in the fluctuations from day to day. The correlation is far from exact, especially as regards the daily variations in milks where the cells were low in number, and the estimation was necessarily subject to relatively large errors, but the general agreement in level is suggestive. The numbers of cells in practically every instance were within the range considered normal for milk from healthy animals, and whereas it has been shown that abnormal numbers of cells tend to inhibit the development of acid by lactic streptococci, the graphs under consideration indicate that, even when allowance is made for variations due to the changes in the vitality of the organisms and for the probable influence of total solid content, the presence of large numbers of cells within the normal range tends to render curd prepared from the milk a better medium for the organisms.

The foregoing tentative conclusion with respect to the influence of cells seemed so important that it was deemed advisable to carry out further experiments with the specific object of confirming or refuting it. Two experiments were therefore performed, the first with six Friesian cows and the second with six Jersey cows. Two of the animals had suffered from mastitis and were still yielding milk containing rather more than the normal numbers of cells, though in no case so many as five millions per cc. Table V gives the relevant particulars

Table V.

Cow	Breed	Average yield of milk per day lbs.	Average fat in milk %	Average total solids in milk %	Days between date of calving and beginning of experiment	Days between recovery from mastitis and beginning of experiment
F 1	Pure Friesian	45	3·0	11·1	93	—
F 2	Pure Friesian	47	3·5	12·2	59	—
F 3	Pure Friesian	49	3·2	11·2	93	—
F 4	Pure Friesian	48	3·3	11·9	91	—
F 5	Pure Friesian	42·5	3·4	11·6	68	—
F 6	Pure Friesian	53	3·6	12·4	95	63
J 1	Grade Jersey	37·5	4·3	13·9	120	—
J 2	Pure Jersey	32·5	6·5	15·9	108	—
J 3	Pure Jersey	33	4·9	14·3	41	—
J 4	Grade Jersey	25	5·5	14·6	201	—
J 5	Pure Jersey	18·5	7·7	17·7	116	—
J 6	Grade Jersey	25	4·5	13·9	94	8

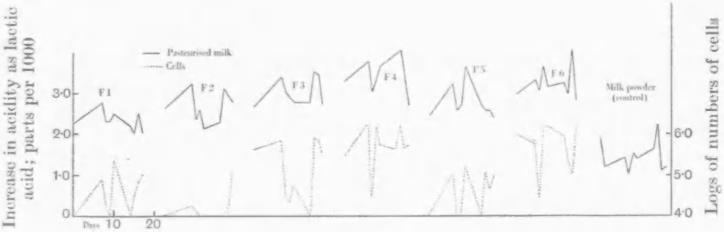

Fig. 2.

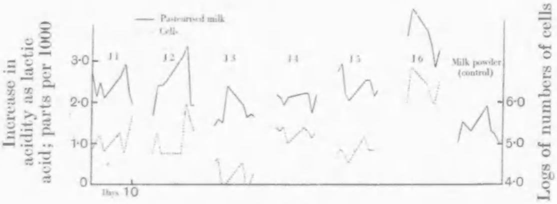

Fig. 3.

of each animal and Figs. 2 and 3 give graphs of the results. On this occasion only the pasteurised milk was tested.

It will be observed that the results of the previous test were substantially confirmed. The Friesian milks again gave a higher general rate of acid production, and perhaps even more definitely in these experiments the rates of acid production showed a correlation with the numbers of cells present in the milk samples. There were one or two anomalies (*e.g.* with the milk from Cow J 4), but in general the correlation was remarkably good. It seemed to make no difference whether the cells were present normally in the milk or were due to recent infection of the udder (*e.g.* in the cases of Cows F 4 and F 6). It seems possible, therefore, that large numbers of cells in the milk of certain cows indicate some previous infection even though no clinical symptoms were ever observed.

The results in any case promise to provide a link, for the first time so far as we are aware, between physiological processes in the cow and the properties of milk as a bacteriological medium. It seems hardly likely that the cells themselves furnish nutriment for the bacteria especially in raw milk where they are in the living state. Furthermore the fact that the correlation between acidity development and cell numbers holds even with milk which has been pasteurised indicates that the stimulating effect is probably due to some chemical entity stable to moderate degrees of heat. The alternative explanation that absence of cells coincides with presence of some inhibitory factor does not seem so satisfactory. Most probably the presence of the stimulating substance in the milk is conditioned by the same physiological influences which affect the numbers of cells. From this aspect the problem appears to be one for the physiologist.

SUMMARY.

1. Milk containing numbers of leucocytes in excess of five millions per cc. gave rise to a rennet curd in which lactic streptococci were not able to develop normal quantities of acid. The influence of the cells was shown to be most probably due to phagocytosis of the streptococci.

2. Cows which were not suffering at the time of the experiments from evident disease of the udder often secreted milk normal in appearance but containing more than five millions of cells per cc.

3. The inhibitory action of the cells was eliminated by a heat treatment of the milk at 49–52° for 30 seconds.

4. The rates of acid production in the rennet curds prepared from the milks of individual healthy cows varied to a significant extent from animal to animal and in one animal from day to day.

5. Some of the differences between milks from different animals were most probably connected with the relative amounts of protein and moisture in the curds.

6. After allowing for the differences due to the factors mentioned under 5 the different rates of acid production appeared to be connected to some extent with the number of cells present in the milks from which the curds were prepared. In most instances investigated the cells were present in numbers not greater than one million per cc. and the higher the number of cells within this range the more rapid was acid production in the curd.

7. The apparent stimulating effect of cells in small numbers in milk upon the growth of lactic streptococci was still manifest after the milk had been pasteurised at 63° for 30 minutes. Therefore, it seems evident that some stimulating substance, stable to moderate degrees of heat, must be secreted in the milk, and its presence must be governed by the same factors which govern the presence of the cells.

We wish to acknowledge our indebtedness to Prof. W. Riddet and Dr F. H. McDowall for much helpful criticism and advice during the course of the work.

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CXXIV. THE ASCORBIC ACID CONTENT OF THE ADRENALS AND LIVERS OF DIFFERENT ANIMALS.

By JOSEPH LOUIS SVIRBELY[1].

From the Institute of Medical Chemistry, University of Szeged, Hungary.

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EXPERIMENTS carried out in this laboratory showed that the ascorbic acid content of the adrenal glands of guinea-pigs decreased markedly if the animals were fed on a vitamin C-free diet. With regard to the biological importance of ascorbic acid it seemed worth while to estimate the ascorbic acid content of different animal organs under normal and varied experimental conditions. This estimation was done by titration with 2 : 6-dibromophenolindophenol blue. The results not only give an idea of the distribution of the acid but at the same time form a test of the reliability of the method of estimation if compared with the known data in literature on the vitamin content of animal organs.

The method was based on the original observation of Szent-Györgyi [1928] that hexuronic acid (ascorbic acid) readily decolorises indophenol blue. The reduction of dichlorophenolindophenol was later developed by Tillmans *et al.* [1932] for a method of estimating vitamin C. Tillmans's method was criticised by Zilva [1932] and later improved by Birch *et al.* [1933].

In the present study 2 : 6-dibromophenolindophenol blue was used which had been standardised against a known solution of ascorbic acid. The estimation was made as follows. The tissue was weighed and minced with sand, and trichloroacetic acid 1·5 % was added until the solution coloured thymol blue slightly red. The extract was filtered and titrated against the indicator to the first permanent trace of pink colour. The values obtained by this method are in fair agreement with biological tests so that on the whole the estimation seems to give reliable results. Glutathione and adrenaline do not disturb the results at this acid reaction. This also is in complete agreement with the results obtained by Birch *et al.* [1933].

It should be emphasised, however, that in exceptional cases the method may be misleading and should be employed critically. In an early paper of Szent-Györgyi [1930] mention is made of a reducing agent in the adrenal medulla.

Another reducing agent which reduces indophenol blue and is not identical with ascorbic acid is found in the juice of the onion. On evacuation of the pressed juice about one-half of its reducing capacity disappears, showing that the entire reduction could not have been due to ascorbic acid which is not volatile.

The cases cited above are those in which the method would give too high values for ascorbic acid and hence also for vitamin C. There are also cases in which without special precautions the method might give much too low values. Many plants contain enzymes which are capable of oxidising the acid very quickly and effectively in the juice or minced pulp. These enzymes are partly or wholly inactive in the uninjured cell. As an example the phenol oxidase may be quoted which, as found by Szent-Györgyi and Vietoris [**1931**], is greatly activated by the process of mincing and will in different plants oxidise ascorbic acid in a few seconds by means of the quinone which is produced. Peroxidase-containing plants, such as cabbage and carrots, also contain enzymes which oxidise ascorbic acid in the juice or minced pulp fairly rapidly in a reversible or irreversible manner. As Tillmans found, the reversible oxidised product is also very labile and prone to undergo irreversible changes. The enzymes of the cabbage have been studied by Szent-Györgyi [1931] and have been termed hexoxidases. Kohman *et al.* [1931] have found that shredding carrots and keeping them for 1–3 hours greatly diminished the vitamin content.

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[1] Holder of an American-Hungarian Exchange Fellowship for 1931–33 from the Institute of International Education, New York.

Apart from these special cases the reduction of indophenol under the described conditions might be taken as a fair estimation of the ascorbic acid content and hence of the vitamin. This method is of value if used extensively for a first orientation but final results should be checked by biological assay.

The results of the present study are given in Table I.

Table I.

Animal	Diet		Mg. ascorbic acid per g. adrenal	Mg. ascorbic acid per g. liver	Remarks
Pig	—		1·22	0·11	Adrenal, whole gland
			1·05	0·13	
			1·23	0·14	
			1·12	0·12	
		Average	1·15	0·12	
Ox	—		0·97	0·40	Adrenal, cortex only
			0·95	0·38	
			0·61	0·27	
			0·53	0·29	
		Average	0·76	0·31	
Calf	—		1·21	0·32	Adrenal, whole gland
			1·05	0·19	
			1·07	0·37	
			0·90	0·30	
			0·97	0·40	
			0·89	0·37	
			0·81	0·40	
		Average	0·99	0·33	
Sheep	—		1·56	0·51	Adrenal, whole gland
			1·35	0·40	
			1·32	0·51	
			1·23	0·46	
			1·39	0·42	
			1·14	0·48	
		Average	1·33	0·46	
Dog	Meat		1·56	0·10	—
			1·48	0·19	
			1·34	0·20	
		Average	1·46	0·16	

Table I (*cont.*).

Animal	Diet	Mg. ascorbic acid per g. adrenal	Mg. ascorbic acid per g. liver	Remarks
Cat	Meat only	1·30	0·18	—
		1·45	0·15	
		1·06	0·19	
		1·07	0·15	
		1·31	0·19	
		1·13	0·17	
	Average	1·22	0·18	
Rabbit	Oats and dried clover	2·22	0·17	This diet was devoid of vitamin C
		2·08	0·07	
		1·41	0·08	
		1·12	0·06	
		3·06	0·13	
		3·08	0·15	
	Average	2·16	0·11	
Rabbit	Oats only for 10 days. Previously on oats and dried clover	2·30	0·11	This diet was devoid of vitamin C
		1·62	0·07	
		1·48	0·08	
		2·85	0·07	
		1·68	0·07	
		1·97	0·09	
	Average	1·83	0·08	
White mouse	Bred for several generations on oats, corn, and moistened bread	3·10	0·38	This diet was devoid of vitamin C
		2·30	0·62	
		2·10	0·42	
		2·20	0·36	
	Average	2·40	0·45	
Rat	Same as for mouse	5·10	0·20	This diet was devoid of vitamin C
		5·30	0·17	
		4·40	0·16	
		6·70	0·20	
		4·80	0·31	
	Average	5·20	0·21	
Guinea-pig	Sherman's basal diet and excess spinach for 3 weeks	1·29	0·32	—
		1·35	0·24	
		1·27	0·26	
		1·14	0·11	
	Average	1·24	0·23	
Guinea-pig	Sherman's basal diet for 3 weeks	0·10	0·02	Severe scurvy in all animals
		0·07	0·02	
		0·06	0·02	
		0·06	0·02	
	Average	0·07	0·02	
Guinea-pig	Sherman's basal diet and excess spinach for 4 days	1·50	0·20	—
		1·70	0·20	
Guinea-pig	Spinach for 4 days and basal diet only for 24 hours	1·60	0·11	—
		1·46	0·09	
Guinea-pig	Spinach for 4 days and basal diet only for 72 hours	0·78	0·04	—
		1·24	0·06	

From this the following conclusions may be drawn.

(1) The ascorbic acid content of the adrenals, based on 1 g. of tissue, is much higher in every animal than that of the liver.

(2) The amount of ascorbic acid in the adrenals and liver is in fair agreement with the known antiscorbutic activities of these organs [Birch *et al.*, 1933; Mills, 1932; Vedder, 1932].

(3) Certain animals, principally the rat and mouse as well as the rabbit, are able to store ascorbic acid in the adrenals and liver even if fed on a vitamin C-free diet. Dogs and cats were likewise able to do so when they were fed on meat which is relatively low in vitamin C. These results are likewise in agreement with that of Birch *et al.*

(4) Guinea-pigs fed first with liberal amounts of spinach and then placed on a vitamin C-free diet showed a decrease in the ascorbic acid content first in the liver and then in the adrenals, indicating that the liver serves as a reserve store for the ascorbic acid.

In order to complete this work an estimation of the ascorbic acid content of the various organs of the guinea-pig was made and the results are given in Table II. As seen from the table the ascorbic acid content is highest in the adrenal and lowest in the muscle, results which are in agreement with previous knowledge.

Table II. *Guinea-pigs fed on sugar beets and basal diet for 2 weeks. No signs of scurvy on autopsy.*

Ascorbic acid in mg./g. of tissue.

					Average
Adrenal	0·18	0·20	0·43	0·19	0·25
Liver	0·016	0·013	0·026	0·02	0·02
Kidney	0·04	0·02	0·02	0·01	0·02
Heart	0·006	0·005	0·007	0·005	0·005
Spleen	0·05	0·08	0·16	0·02	0·08
Leg muscle	0·001	0·002	0·01	0·001	0·005

SUMMARY.

A study is made of the reducing capacity, and hence of the ascorbic acid content, of the adrenals and livers of different animals. The technique of estimating the ascorbic acid is described and discussed.

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